Remarks

Claims 43-54 are pending. Claims 50-54 have been withdrawn by the U.S. Patent and Trademark Office (hereinafter "the Patent Office"). Claims 43-49 have been examined by the Patent Office and presently stand rejected.

Claims 43-44 and 47-48 have been rejected under 35 U.S.C. § 102(b) upon the contention that the claims are anticipated by U.S. Patent No. 5,334,380 to Kilbourn et al. (hereinafter "Kilbourn et al.").

Claims 43-49 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over McCaffrey et al. (1995 *Biol. Neonate* 67:240-243; hereinafter "McCaffrey et al.") in view of U.S. Patent No. 5,767,160 to Kaesemeyer et al. (hereinafter "Kaesemeyer et al.").

Claims 43-44 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Kaesemeyer et al.

Claims 45-49 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Kaesemeyer et al. in view of McCaffrey et al.

Claims 43 and 50 have been amended to more particularly recite the presently disclosed subject matter. Support for the amendments can be found throughout the specification as filed, including particularly in claims 43 and 50 as filed. No new matter has been added.

Reconsideration of the application based on the amendments and arguments set forth herein is respectfully requested.

II. Information Disclosure Statement

The Patent Office contends that the Information Disclosure Statement submitted on March 17, 2011, fails to comply with the provisions of 37 CFR §§ 1.97, 1.98 and MPEP § 609 because it allegedly provides incomplete citations for reference citation numbers 49-68 and 72. Without acquiescing to the contentions of the Patent Office, applicants respectfully submit that a Supplemental Information Disclosure Statement is submitted herewith. In the Supplemental Information Disclosure Statement full citations

are provided for each of the references corresponding to reference citation numbers 49-68 and 72 in the March 17, 2011 Information Disclosure Statement.

The Patent Office also contends that the Information Disclosure Statement submitted on June 1, 2011, fails to comply with the provisions of 37 CFR § 1.98(a)(2). The Patent Office contends that copies of documents corresponding to reference citation numbers 19 and 82 were not provided. In response, applicants respectfully submit that reference citation number 19 (Castillo et al.) in the Information Disclosure Statement submitted on June 1, 2011, was an inadvertent and incorrect reference citation. No such reference is believed to exist. Reference citation number 19 in the Information Disclosure Statement submitted on June 1, 2011 can be omitted from the record. Of note, reference citation number 18 in the Information Disclosure Statement submitted on June 1, 2011, also to Castillo et al., is believed to be correct.

As for reference citation number 82 (Schwartz et al.), applicants respectfully submit that this citation was also inadvertently included in the Information Disclosure Statement submitted on June 1, 2011. This reference is a general background reference that is believed to be cumulative of the art of record and therefore not material. Reference citation number 82 in the Information Disclosure Statement submitted on June 1, 2011 can therefore also be omitted from the record.

III. Election/Restrictions

In the instant Official Action claims 50-54 are indicated as withdrawn from further consideration upon the contention that the claims are drawn to non-elected species (elected species is pulmonary hypertension). Thus, claims 43-49 have been examined on the merits.

Applicants respectfully submit that claim 50 has been amended herein. In particular, present claim 50 recites a method of treating or preventing pulmonary hypertension comprising administering an amount of citrulline effective to treat or prevent pulmonary hypertension in a subject, wherein the subject is exposed to or about to be exposed to cardiac surgery, and whereby low postoperative pulmonary vascular tone is maintained. Support for the amendment can be found throughout the

specification as filed, including particularly in claim 50 as filed. No new matter has been added.

Applicants respectfully submit that as a result of the present amendment, independent claim 50, and dependent claims 51-54 depending therefrom, clearly include the elected species, and therefore should also be examined on the merits.

IV. Response to the 35 U.S.C. § 102(b) Rejection of Claims 43-44 and 47-48 Based on Kilbourn et al.

Claims 43-44 and 47-48 presently stand rejected under 35 U.S.C. § 102(b) upon the contention that the claims are anticipated by Kilbourn et al. The Patent Office contends that Kilbourn et al. discloses the intravenous administration of citrulline to an animal subject, including a human patient. The Patent Office further contends that the preamble limitation of "treating or preventing pulmonary hypertension" is not accorded any patentable weight. Moreover the Patent Office contends that the preamble is an inherent characteristic of the method. Therefore, the Patent Office contends that Kilbourn et al. allegedly discloses a method that inherently meets the requirements of claims 43-44 and 47-48.

After careful consideration of the rejection and the Patent Office's basis therefore, applicants respectfully traverse the rejection and submit the following remarks.

Initially, without acquiescing to the contentions of the Patent Office, applicants respectfully submit that claim 43 has been amended. In particular, present claim 43 recites a method of treating or preventing pulmonary hypertension comprising administering an amount of citrulline effective to treat or prevent pulmonary hypertension in a subject in need thereof, wherein said citrulline is administered intravenously. Support for the amendment can be found throughout the specification as filed, including particularly in claim 43 as filed. No new matter has been added.

It is well settled that for a cited reference to qualify as prior art under 35 U.S.C. §102, each element of the claimed invention must be disclosed within the reference. "A claim is anticipated only if each and every element as set forth in the claim is found,

either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). *See also* M.P.E.P. § 2131. Applicants respectfully submit that Kilbourn et al. does not support a rejection of claims 43-44 and 47-48 because each and every element as set forth in the claims is not found, either expressly or inherently described, in Kilbourn et al.

To elaborate, present claim 43 is directed to a method of treating or preventing pulmonary hypertension comprising administering to a subject an amount of citrulline effective to treat or prevent pulmonary hypertension. Applicants respectfully submit that Kilbourn et al. fails to teach a method of treating or preventing pulmonary hypertension. In marked contrast, Kilbourn et al., at best, provides for the administration of citrulline to a treat or prevent "hypotension". *See*, e.g., the Abstract and column 1, line 19, of Kilbourn et al. Hypotension is not equivalent to hypertension.

Hypotension is a physiologic condition distinct from hypertension. Hypotension is defined as "[l]ow blood pressure...when blood pressure during and after each heartbeat is much lower than usual." *See* **Exhibit D** provided herewith, also found at http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0004536/ (of note, Exhibits A, B and C were previously submitted with the response filed February 28, 2011). Conversely, hypertension is defined as a "term used to describe high blood pressure." *See* **Exhibit E** provided herewith, also found at http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001502/.

As such, a population of patients having hypotension, and treated by Kilbourn et al., is distinct and separate from a population having hypertension, as presently claimed. Indeed, treatment according to by Kilbourn et al. to deal with hypotension, by increasing blood pressure will, if anything, cause hypertension rather than preventing it. Nowhere does Kilbourn et al. disclose or suggest treating pulmonary hypertension. As such, Kilbourn et al. is not an anticipatory reference and cannot support a rejection of present claim 43 under 35 U.S.C. § 102(b).

Additionally, the claim 43 has now been amended to recite "an amount effective to treat or prevent pulmonary hypertension," which ties the preamble to the body of the

claim. Accordingly, applicants respectfully submit that Kilbourn et al. does not anticipate the claimed methods.

As such, applicants respectfully submit that independent claim 43 has been distinguished over Kilbourn et al. Applicants further submit that claims 44 and 47-48 ultimately depend from independent claim 43. As such, applicants respectfully submit that the rejection of these claims has been addressed as well. Accordingly, applicants respectfully request that the instant rejection of claims 43-44 and 47-48 under 35 U.S.C. § 102(b) be withdrawn at this time. A Notice of Allowance is also respectfully requested.

Of note, applicants respectfully submit that claim 50, which has been amended to recite, *inter alia*, a method of treating or preventing pulmonary hypertension comprising administering an amount of citrulline effective to treat or prevent pulmonary hypertension in a subject, is also believed to be distinguished from Kilbourn et al. Dependent claims 51-54 are believed to be allowable for at least the same reasons.

V. Response to the 35 U.S.C. § 103(a) Rejection of Claims 43-49 Based on McCaffrey et al. in view of Kaesemeyer et al.

Claims 43-49 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over McCaffrey et al. in view of Kaesemeyer et al. The Patent Office contends that McCaffrey et al. discloses treating infants with a persistent pulmonary hypertension of the newborn (PPHN) via an intravenous infusion of 500 milligrams per kilogram L-arginine. The Patent Office admits that McCaffrey et al. do not teach the use of citrulline in place of arginine. However, the Patent Office contends that Kaesemeyer et al. teaches the use of arginine and its "biological equivalent" citrulline for the treatment of pulmonary hypertension. As such, the Patent Office contends that it would have been *prima facie* obvious to one of ordinary skill in the art to have substituted citrulline for arginine in the method of McCaffrey et al.

After careful consideration of the rejection and the Patent Office's basis therefore, applicants respectfully traverse the rejection and submit the following remarks.

McCaffrey et al., at best, teaches the treatment of pulmonary hypertension in newborns comprising administering intravenous L-arginine. McCaffrey et al. does not teach a method of treating or preventing pulmonary hypertension comprising administering intravenous amounts of citrulline effective to treat or prevent pulmonary hypertension in a subject, as claimed in present claim 43. This deficiency is not remedied by Kaesemeyer et al.

Kaesemeyer et al. teaches the administration of a combination of L-arginine and an agonist of NO synthase (i.e., nitroglycerin) to treat diseases related to vasoconstriction. Kaesemeyer et al. also suggests that citrulline is a "biological equivalent" of L-arginine. *See* column 2, lines 22–23. However, Kaesemeyer et al. fails to substantiate through testing that there is, in fact, equivalence between the two molecules. At best, it is a suggestion, and that suggestion is incorrect. There is an abundance of evidence that these two molecules, i.e. arginine and citrulline, play functionally different roles metabolically and in the production of nitric oxide, and these differences would come as a surprise to anyone who believed that these two molecules are equivalent.

Prior to the disclosure of the presently disclosed and claimed subject matter, intravenous administration of citrulline was not studied in human subjects. *See*, **Exhibit B** (Barr et al., *J of Thoracic and Cardiovascular Surgery*, August 2007, 134(2):319; previously submitted with the response filed February 28, 2011, but provided again herewith as a courtesy). Studies using intravenous citrulline were most likely not conducted because intravenous formulations of citrulline were not available. *See,* **Exhibit F** (Summar, *J. of Pediatrics*, 2001, 138(1):S30-S39, S35) provided herewith. Thus, distinctions between effects of citrulline and arginine could (and did) pass unnoticed.

However, in studies where L-arginine and citrulline were supplied at the level of endothelial cells, surprisingly, "added arginine did not cause as great an increase in endothelial NO production as did added citrulline." *See* Solomonson et al., *J. Exp. Biol.*, 2003, 206:2083-2087, p. 2085, col. 1, lines 3-8, provided herewith as **Exhibit G**. "Furthermore, the effects of arginine and citrulline on NO production appeared to be

synergistic, since a combination of arginine and citrulline stimulated endothelial NO production more than did either arginine or citrulline alone." *Id.*, lines 17-20. Thus, citrulline does not simply act as an equivalent replacement for arginine. These authors also noted that "[a]lthough supplemental arginine can be beneficial in some cases, in other cases it may lead to adverse effects owing to the multiple metabolic roles of arginine." *Id.*, at 2084, col. 2, lines 13-16, citations omitted. Consequently, using citrulline rather than arginine therapeutically has the potential to avoid some adverse effects, "[s]ince arginine has a number of potential metabolic fates, while citrulline has only one known metabolic fate." *Id.* at 2085, col. 1, lines 21-23 and Fig. 1.

Moreover, the present inventors have demonstrated that citrulline treatment is effective in an pig model of pulmonary hypertension. *See* **Exhibit C** (Anathakrishnan et al., *Am J. Physiol Lung cell Mol Physiol*, 2009 297:L506-L511, L509, col. 2, lines 4-16; previously submitted with the response filed February 28, 2011, but provided again herewith as a courtesy).

In view of the evidence now of record, citrulline is clearly not equivalent to arginine. Thus, upon consideration of all of the evidence, the *prima facie* case of obviousness proposed in the instant Official Action must fail. Furthermore, testing such as that reported by Solomonson et al. (**Exhibit G**), discussed above, provides results which would be surprising in view of the *prima facie* case of obviousness proposed by the Patent Office in the instant Official Action. Therefore, the evidence provided herewith would rebut the instant obviousness rejection, and the rejection should be withdrawn.

Taken together, applicants respectfully submit that the instant 35 U.S.C. §103(a) rejection of claims 43-49 as allegedly being unpatentable over McCaffrey et al. in view of Kaesemeyer et al. has been addressed. Accordingly, applicants respectfully request that the rejection of claims 43-49 be withdrawn at this time. A Notice of Allowance directed to these claims is also respectfully requested.

Of note, applicants respectfully submit that claim 50, which has been amended to recite, *inter alia*, a method of treating or preventing pulmonary hypertension comprising administering an amount of citrulline effective to treat or prevent pulmonary

hypertension in a subject, is also believed to be patentable over McCaffrey et al. in view of Kaesemeyer et al. for at least the same reasons. Dependent claims 51-54 are believed to be allowable for at least the same reasons.

VI. Response to the 35 U.S.C. § 103(a) Rejection of Claims 43-44 Based on Kaesemeyer

Claims 43-44 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Kaesemeyer et al. The Patent Office contends that Kaesemeyer et al. teaches the use of arginine and its "biological equivalent" citrulline in the treatment of pulmonary hypertension in any mammalian subject. As such, the Patent Office contends that it would have been *prima facie* obvious to one of ordinary skill in the art to have substituted citrulline for arginine in the method of Kaesemeyer et al.

After careful consideration of the rejection and the Patent Office's basis therefore, applicants respectfully traverse the rejection and submit the following remarks.

Applicants respectfully direct the Patent Office's attention to the discussion hereinabove regarding substituting citrulline for arginine, as well as the deficiencies in Kaesemeyer et al. with respect to the presently disclosed and claimed subject matter. As discussed above, claims 43-44 are not *prima facie* obvious over the proposed combination of McCaffrey et al. and Kaesemeyer et al. For at least the same reasons claims 43-44 are not *prima facie* obvious over Kaesemeyer et al. when taken alone.

Applicants respectfully submit that the instant 35 U.S.C. §103(a) rejection of claims 43-44 as allegedly being unpatentable over Kaesemeyer et al. has been addressed. Accordingly, applicants respectfully request that the rejection of claims 43-44 be withdrawn at this time. A Notice of Allowance directed to these claims is also respectfully requested.

Of note, applicants respectfully submit that present claim 50 is also believed to be patentable over Kaesemeyer et al. for at least the same reasons. Dependent claims 51-54 are believed to be allowable for at least the same reasons.

VII. Response to the 35 U.S.C. § 103(a) Rejection of Claims 45-49 Based on Kaesemeyer et al. in view of McCaffrey et al.

Claims 45-49 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Kaesemeyer et al. in view of McCaffrey et al.

After careful consideration of the rejection and the Patent Office's basis therefore, applicants respectfully traverse the rejection and submit the following remarks.

Initially, applicants respectfully note that this rejection appears to be duplicative of the § 103(a) rejection above over McCaffrey et al. in view of Kaesemeyer et al., with the only difference being the order of the cited references in the proposed combination.

Nevertheless, applicants respectfully direct the Patent Office's attention to the discussion hereinabove regarding substituting citrulline for arginine, as well as the deficiencies in McCaffrey et al. and Kaesemeyer et al. with respect to the presently disclosed and claimed subject matter. As discussed above, claims 43-49 are not *prima facie* obvious over the proposed combination of McCaffrey et al. and Kaesemeyer et al. For at least the same reasons claims 45-49 are not *prima facie* obvious over Kaesemeyer et al. in view of McCaffrey et al.

Applicants respectfully submit that the instant 35 U.S.C. §103(a) rejection of claims 45-49 as allegedly being unpatentable over Kaesemeyer et al. in view of McCaffrey et al. has been addressed. Accordingly, applicants respectfully request that the rejection of claims 45-49 be withdrawn at this time. A Notice of Allowance directed to these claims is also respectfully requested.

Of note, applicants respectfully submit that present claim 50 is also believed to be patentable over Kaesemeyer et al. in view of McCaffrey et al. for at least the same reasons. Dependent claims 51-54 are believed to be allowable for at least the same reasons.

CONCLUSION

Should there be any minor issues outstanding in this matter, the Examiner is respectfully requested to telephone the undersigned attorney. Early passage of the subject application to issue is earnestly solicited.

DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any fees associated with the filing of this correspondence to Deposit Account Number **50-0426**.

Respectfully submitted,

JENKINS, WILSON, TAYLOR & HUNT, P.A.

Date: 03/27/2012

By: 

Arles A. Taylor, Jr.
Registration No. 39,395
(919) 493-8000
Customer No. 25297

1242/58 AAT/LRL/dbp

Pharmacokinetics and safety of intravenously administered citrulline in children undergoing congenital heart surgery: Potential therapy for postoperative pulmonary hypertension

Frederick E. Barr, MD, MSci,[a] Rommel G. Tirona, PhD,[b] Mary B. Taylor, MD, MSci,[a] Geraldine Rice, RN, BSN,[a] Judith Arnold, RN, BSN,[a] Gary Cunningham, MS,[a] Heidi A. B. Smith, MD, MSci,[a] Adam Campbell, BS,[a] Jeffrey A. Canter, MD, MPH,[e] Karla G. Christian, MD,[c] Davis C. Drinkwater, MD,[c] Frank Scholl, MD,[c] Ann Kavanaugh-McHugh, MD,[d] and Marshall L. Summar, MD[e]

Objective: Pulmonary hypertension may complicate surgical correction of congenital heart defects, resulting in increased morbidity and mortality. We have previously shown that plasma levels of the nitric oxide precursors citrulline and arginine drop precipitously after congenital cardiac surgery and that oral citrulline supplementation may be protective against the development of pulmonary hypertension. In this study, we assessed the safety and pharmacokinetic profile of intravenous citrulline as a potential therapy for postoperative pulmonary hypertension.

Methods: The initial phase of this investigation was a dose-escalation study of intravenously administered citrulline in infants and children undergoing one of five congenital cardiac surgical procedures (phase 1). The primary safety outcome was a 20% drop in mean arterial blood pressure from the baseline pressure recorded after admission to the intensive care unit. Based on our previous work, the target circulating plasma citrulline trough was 80 to 100 μmol/L. Each patient was given two separate doses of citrulline: the first in the operating room immediately after initiation of cardiopulmonary bypass and the second 4 hours later in the pediatric intensive care unit. Stepwise dose escalations included 50 mg/kg, 100 mg/kg, and 150 mg/kg. After model-dependent pharmacokinetic analysis, we enrolled an additional 9 patients (phase 2) in an optimized dosing protocol that replaced the postoperative dose with a continuous infusion of citrulline at 9 mg/(kg · h) for 48 hours postoperatively.

Results: The initial stepwise escalation protocol (phase 1) revealed that an intravenous citrulline dose of 150 mg/kg given after initiation of cardiopulmonary bypass yielded a trough level of in the target range of approximately 80 to 100 μmol/L 4 hours later. The postoperative dose revealed that the clearance of intravenously administered citrulline was 0.6 L/(h · kg), with a volume of distribution of 0.9 L/kg and estimated half-life of 60 minutes. Because of the short half-life, we altered the protocol to replace the postoperative dose with a continuous infusion of 9 mg/(kg · h). An additional 9 patients were studied with this continuous infusion protocol (phase 2). Mean plasma citrulline levels were maintained at approximately 125 μmol/L, with a calculated clearance of 0.52 L/(h · kg). None of the 17 patients studied had a 20% drop in mean arterial blood pressure from baseline.

Conclusions: In this first report of the use of intravenous citrulline in humans, we found citrulline to be both safe and well tolerated in infants and young children undergoing congenital cardiac surgery. Because of the rapid clearance, the optimal dosing regimen was identified as an initial bolus of 150 mg/kg given at the initiation of cardiopulmonary bypass, followed 4 hours later by a postoperative infusion of 9 mg/(kg · h) continued up to 48 hours. Using this regimen, plasma arginine, citrulline, and nitric oxide metabolite levels were well maintained. Intravenous citrulline needs to be studied further as a potential therapy for postoperative pulmonary hypertension.

From the Departments of Pediatrics, Pediatric Critical Care,[a] Pharmacology,[b] Cardiothoracic Surgery,[c] and Pediatric Cardiology,[d] and the Center for Human Genetics Research,[e] Vanderbilt Children's Hospital, Vanderbilt University Medical Center, Nashville, Tenn.

Supported by the National Institutes of Health, National Heart, Lung, and Blood Institute, grant RO1 HL073317 (F.E.B.).

Received for publication Oct 27, 2006; revisions received Jan 11, 2007; accepted for publication Feb 1, 2007.

Address for reprints: Frederick E. Barr, MD, MSci, 2200 Children's Way, 5121 Doctor's Office Tower, Nashville TN 37232-9075 (E-mail: rick.barr@vanderbilt.edu).

J Thorac Cardiovasc Surg 2007;134:319-26

0022-5223/$32.00

doi:10.1016/j.jtcvs.2007.02.043

CHD

Pulmonary hypertension is a potential complication after surgical correction of congenital cardiac defects that has been associated with increased postoperative morbidity and mortality.[1-5] Current perioperative treatment includes the use of inhaled nitric oxide and several nonselective pulmonary vasodilators, including milrinone, epoprostenol, sildenafil citrate (INN sildenafil), and generation of alkalosis. Another potential therapy includes increasing endogenous nitric oxide synthesis by supplementation with citrulline or arginine.[6,7] Nitric oxide is produced from L-citrulline and L-arginine, amino acids generated through the urea cycle (Figure 1).[8-10] We have previously demonstrated that citrulline and arginine levels drop precipitously after surgical correction of congenital cardiac defects with cardiopulmonary bypass and do not recover for as long as 48 hours after surgery.[11] In addition, children with postoperative pulmonary hypertension had significantly lower plasma arginine levels than those without this complication. We subsequently conducted a randomized, placebo-controlled trial of oral citrulline supplementation in this same patient population and found that although absorption was variable, citrulline was safe and that children who had a plasma citrulline level greater than 40 μmol/L at 12 hours after surgery were free from postoperative pulmonary hypertension.[10]

Intravenously administered citrulline has not been previously studied in human beings. The hypothesis of this study was that intravenously administered citrulline would be safe and would prevent the postoperative drop in plasma citrulline and arginine levels noted in our previous observational studies.

Materials and Methods

Patient Enrollment

Approval from Vanderbilt's institutional review board (IRB) was obtained before patient enrollment. A total of 17 patients were enrolled in this open-label dose-escalation study at Vanderbilt Children's Hospital between May 2005 and January 2006.

All infants or children younger than 6 years undergoing one of five surgical procedures for correction of congenital heart lesions were considered for enrollment. The eligible surgical procedures were as follows: (1) repair of atrioventricular septal defect, (2) repair of ventricular septal defect, (3) bidirectional Glenn procedure (superior cavopulmonary shunt), (4) modified Fontan procedure (total cavopulmonary connection), and (5) arterial switch procedure. Exclusion criteria were as follows: (1) significant pulmonary arterial narrowing not addressed surgically, (2) previous pulmonary artery stent placement, (3) previous pulmonary artery angioplasty, (4) significant left-sided atrioventricular valve regurgitation, (5) pulmonary venous return abnormalities, (6) pulmonary vein stenosis, (7) preoperative mechanical ventilation, and (8) preoperative inotropic infusions.

Informed written consent was obtained from parents of the enrolled patients during preoperative evaluation at the Cardiothoracic Surgery Clinic (outpatient) or at Vanderbilt Children's Hospital (inpatient). Three cardiac surgeons at Vanderbilt Children's Hospital (K.G.C., D.C.D., F.S.) performed the surgical procedures with similar cardiopulmonary bypass and cardioplegia preparations.

Abbreviations and Acronyms

DSMB = data safety monitoring board
FDA = Food and Drug Administration
IRB = institutional review board
k_{cern} = constant of citrulline removal
PICU = pediatric intensive care unit
R_{app} = rate of citrulline appearance

This study was monitored closely by a data safety monitoring board (DSMB) composed of a pediatric cardiologist, a pediatric critical care physician, and a general pediatrician. The DSMB met three times during the study to review the safety and pharmacokinetic data.

Adverse Events

Intravenous citrulline administration carries a theoretic risk of systemic arterial hypotension. An adverse drop in mean arterial pressure was defined as a decrease of more than 20% from baseline. The baseline postoperative mean arterial blood pressure was calculated as the average of mean arterial blood pressure measurements collected every 5 minutes for the 30 minutes immediately before the administration of the postoperative dose or infusion. The bedside monitor was then set to alarm if that 20% drop was reached at any time in the 48-hour study period. If the bedside monitor reached the preset limit and alarmed, the beside nurse was instructed to alert the study physician or nurse and to record mean arterial pressure every 5 minutes for 30 minutes. If the average of these 5-minute recordings was 20% below the original baseline blood pressure, the citrulline was discontinued. Patients were treated for hypotension at the discretion of the clinical pediatric intensive care unit (PICU) staff with volume resuscitation, inotropic or vasopressor support, or both. Development of hypotension according to these criteria that necessitated discontinuation of citrulline was counted as an adverse event and was reported to the DSMB, IRB, and Food and Drug Administration (FDA).

Serious adverse events, such as cardiac arrest, need for extracorporeal membrane oxygenation, and death, were reported immediately to the DSMB, the Vanderbilt IRB, and the FDA.

Study Protocol

The study design for the first 8 patients (phase 1) was a dose-escalation protocol with two intravenously administered bolus doses of citrulline to determine the optimal dose and characterize pharmacokinetic parameters including half-life, clearance, and volume of distribution. The first bolus (given during the course of 10 minutes) was administered after initiation of cardiopulmonary bypass in the operating room; and the second (given during the course of 30 min) was administered 4 hours later in the critical care unit. The intravenous doses of citrulline for each bolus were 50 mg/kg (2 patients), 100 mg/kg (2 patients), and 150 mg/kg (4 patients).

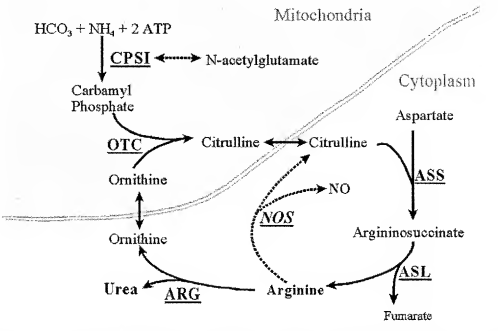


Figure 1. Carbamyl phosphate synthetase I *(CPSI)* is rate-limiting enzyme of urea cycle. Polymorphisms in its gene alter availability of nitric oxide precursors, citrulline, and arginine. Citrulline is metabolized into arginine, which is then metabolized into either nitric oxide *(NO)* by nitric oxide synthetase *(NOS)* or urea by arginase *(ARG)*. HCO_3, Bicarbonate; NH_4, ammonium; *ATP*, adenosine triphosphate; *OTC*, ornithine transcarbamylase; *ASS*, argininosuccinate synthetase; *ASL*, argininosuccinate lyase. From Smith HA, Canter JA, Christian KG, Drinkwater DC, Scholl FG, Christman BW, et al. Nitric oxide precursors and congenital heart surgery: a randomized controlled trial of oral citrulline. *J Thorac Cardiovasc Surg*. 2006;132:58-65.

The study design was changed (phase 2) after it was determined that the half-life and clearance were not compatible with intermittent dosing. After consultation with a pharmacologist, the next 9 patients received a 150-mg/kg intravenously administered bolus of citrulline during the course of 10 min in the operating room after initiation of cardiopulmonary bypass, followed 4 hours later in the critical care unit by a continuous infusion of 9 mg/(kg · h) that continued for 48 hours.

The citrulline preparation was provided by the Investigational Drug Service of the Vanderbilt Hospital Clinical Pharmacy. Citrulline was administered as a 50-mg/mL (5%) isotonic solution, with distilled water as a suspending agent.

Sample Collection

A 3-mL sample of blood was obtained from each patient at selected time points. In phase 1, samples were collected immediately after initiation of cardiopulmonary bypass before the first bolus given in the operating room, postoperatively 4 hours after the operating room bolus (immediately before the postoperative bolus), and then 1, 2, 3, 4, and 12 hours after the postoperative bolus. In phase 2, samples were collected immediately after initiation of cardiopulmonary bypass (before the first bolus given in the operating room), immediately after the operating room bolus, postoperatively 4 hours after the operating room bolus (immediately before the postoperative infusion), and then 6, 12, 24, and 48 hours after initiation of the postoperative continuous infusion. Samples were collected in citrated tubes, placed on ice, and stored at 4°C until processing. Samples were centrifuged for separation of plasma and cellular components. Plasma samples were frozen at −70°C until further laboratory analysis.

Laboratory Measurements

Concentrations of plasma citrulline and arginine were determined through amino acid analysis by cation-exchange chromatography with a Beckman 7300 amino acid analyzer (Beckman Coulter, Inc, Fullerton, Calif). Calibration of the analyzer with known standards was completed before testing of patient samples.

Nitric oxide metabolites were measured by chemiluminescence with a Sievers 280 nitric oxide analyzer (GE Analytical Instruments, Boulder, Colo). Plasma samples were mixed 1:2 sample/cold ethanol at 0°C for 30 minutes. After centrifugation at 14,000 rpm for 5 minutes, samples were injected into the analyzer. This method relies on catalytic reduction of nitric oxide metabolites by exposure to warm vanadium hydrochloride. Liberated nitric oxide was driven by nitrogen gas into an ozone chamber. Light released by nitric oxide–ozone interaction was captured by a photomultiplier tube and relayed to an analytic software program. A standard curve with sodium nitrite was used to determine sample concentrations.

CHD

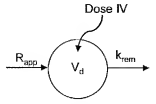


Figure 2. Pharmacokinetic model used in analysis of phase 1 data and for design of phase 2 intravenous *(IV)* dosing regimen. In this model, body is represented as single compartment with volume of distribution (V_d). Citrulline appearance in plasma is described by zero-order process (R_{app}) to account for endogenous production, whereas removal from plasma is determined by first-order process (k_{rem}).

Pharmacokinetic Analysis

Data obtained for each patient in phase 1 were fitted to the pharmacokinetic model depicted in Figure 2. In this model, the body exists as a single compartment with a volume of distribution. The appearance of citrulline in plasma is described by a zero-order process (rate of citrulline appearance, R_{app}) to account for endogenous production, whereas the removal of citrulline is determined by a first-order process (constant of citrulline removal, k_{rem}). It is assumed that the values of all parameters remained constant for each patient during the course of plasma sampling. Mass–balance differential equations were inputted into SCIENTIST (MicroMath Scientific Software, St Louis, Mo) and solved numerically. Data fitting was accomplished by a weighted, least squares procedure to obtain the simultaneous estimates of R_{app}, k_{rem}, and volume of distribution. Clearance was calculated from k_{rem} multiplied by the volume of distribution, and half-life was estimated as $\ln 2/k_{rem}$.

Results

Patient Enrollment

Seventeen patients were successfully enrolled, 8 patients in phase 1 and 9 in phase 2. The median age of the patients was 6 months (interquartile range 3.6–30.6 months), with 55% male and 81% white. Surgical interventions were as follows: 4 patients with ventricular septal defect repair, 8 patients with atrioventricular septal defect repair, 2 patients with bidirectional Glenn shunt, 1 patient with modified Fontan procedure, and 2 patients with arterial switch.

Safety

There were no significant adverse events in phase 1. There was 1 significant adverse event in phase 2. That patient underwent an atrioventricular septal defect repair and was in a junctional rhythm postoperatively, necessitating atrial pacing. At approximately 8 postoperative hours, the patient showed the acute onset of profound bradycardia consistent with complete heart block that was not preceded by systemic hypotension and was not responsive to ventricular pacing. Advanced life-support measures were instituted, including open cardiac massage and emergency cannulation for venoarterial extracorporeal membrane oxygenation, which was required for 48 hours. The patient subsequently recovered fully and was discharged home on hospital day 22. The DSMB reviewed the case and determined that the significant adverse event was unlikely to be related to the citrulline administration. The adverse event was also reported to the Vanderbilt IRB, the National Institutes of Health, and the FDA.

Pharmacokinetics

Phase 1. Patients in phase 1 were given two doses of intravenously administered citrulline, the first just after initiation of cardiopulmonary bypass and the second 4 hours later after admission to the PICU. The plasma levels of citrulline at the various times after drug administration are depicted in Figure 3. Patients 1 and 2 received 50-mg/kg citrulline intravenously and had a peak citrulline level of approximately 220 μmol/L and a 4-hour trough level of 40 μmol/L. No adverse side effects were noted. This trough was well below the target range of 80 to 100 μmol/L, and the dose was subsequently increased. Patients 3 and 4 received 100-mg/kg citrulline intravenously and had a peak citrulline level of 375 μmol/L and a 4-hour trough of 50 μmol/L. Again, no adverse side effects were noted. This trough was also below the target range of 80 to 100 μmol/L, and the dose was subsequently increased. Patient 5, 6, 7, and 8 received 150 mg/kg citrulline intravenously and had a peak citrulline level of 660 μmol/L and a 4-hour trough of 80 μmol/L. This 4-hour trough was in the target range of 80 to 100 μmol/L, and the dose was not escalated further. The citrulline pharmacokinetic parameter estimates for each of the three dosage levels are summarized in Table 1, and the pharmacokinetic profiles are displayed in Figure 3. The half-life was calculated to be approximately 60 minutes, which was too short to proceed with intermittent dosing. The study design was changed to a bolus dose followed by a continuous infusion in phase 2.

Phase 2. An additional 9 patients were enrolled in phase 2. On the basis of parameter estimates obtained from phase 1 (Table 1), pharmacokinetic simulations predicted that a bolus dose of 150 mg/kg followed 4 hours later by a continuous infusion of 9 mg/(kg · h) would yield sustained plasma citrulline levels of approximately 80 to 100 μmol/L (Figure 4). Initiation of the continuous infusion was deliberately set at 4 hours after the bolus to allow sustained increased levels during separation from cardiopulmonary bypass and ultrafiltration, to allow time for admission to the PICU, and to allow postoperative hemodynamic stabilization to allow accurate assessment of the drug safety profile. The phase 2 mean plasma citrulline profile is shown in Figure 5. For the entire group, postoperative mean plasma citrulline levels were sustained at approximately 150 to 250

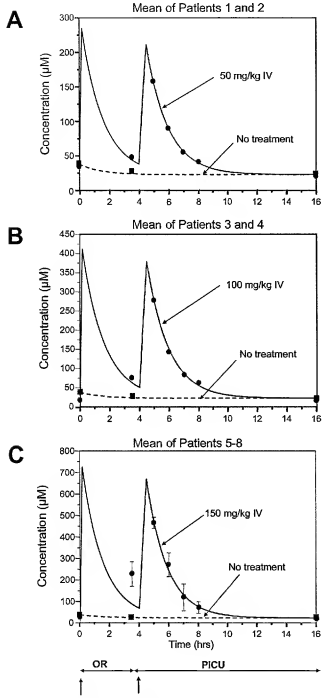


Figure 3. Pharmacokinetic profile of intravenously administered *(IV)* bolus-dose citrulline in phase 1. Dose-escalation study design in which each patient was given two boluses of citrulline, first in operating room *(OR)* on cardiopulmonary bypass and second 4 hours later after admission to pediatric intensive care unit *(PICU)*. Patients 1 and 2 received 50 mg/kg (A), patients 3 and 4 received 100 mg/kg (B), and patients 5, 6, 7, and 8 received 150 mg/kg (C). No adverse side effects were noted.

μmol/L during the 48-hour study period. Estimated citrulline clearance was 0.52 ± 0.28 L/(kg · h) in phase 2. The mean plasma citrulline levels of a previously published cohort of patients undergoing congenital cardiac surgery who had not received intravenously administered citrulline are also depicted in Figure 5 for comparison.[11] Figure 6 depicts similar data for plasma arginine levels, which were also sustained after intravenous administration of citrulline relative to data from the previous cohort.[11] Figure 7 depicts similar data for plasma nitric oxide metabolite levels, which were also sustained after intravenous administration of citrulline relative to data from the previous cohort.[11]

Discussion

This is the first report of the use of intravenously administered citrulline in human beings. The goal of this study was to determine the safety profile and pharmacokinetics of intravenously administered citrulline in a fairly unique patient population of infants and children undergoing surgical correction of congenital heart defects. This was an open-label study that was not placebo controlled and thus not designed to evaluate the use of intravenously administered citrulline as a potential therapy for postoperative pulmonary hypertension. The pharmacokinetic and safety information gained from this study, however, has been subsequently used to design a randomized, placebo-controlled efficacy trial of intravenously administered citrulline for the treatment of postoperative pulmonary hypertension.

With regard to safety, we were primarily interested in systemic hypotension as a potential negative side effect of citrulline treatment. We defined systemic hypotension as a sustained 20% drop in mean arterial pressure from an average baseline recording that was obtained during the 30 minutes just before initiation of intravenous citrulline administration, either as a bolus (phase 1) or as a continuous infusion (phase 2). This definition took into account some of the inherent blood pressure variation in children immediately after congenital cardiac surgery. None of the patients in phase 1 met this definition of systemic hypotension. Only 1 patient in phase 2 of this study met this definition, and that patient's hypotension was precipitated by profound bradycardia and heart block, a side effect unlikely to be precipitated by citrulline. The DSMB reviewed that patient's clinical data and determined that the adverse event was unlikely to be related to the citrulline infusion. The Vanderbilt IRB, the National Institutes of Health, and the FDA were also notified and concurred with this conclusion.

The pharmacokinetics of intravenously administered citrulline in this patient population are significantly complicated by the interposition of cardiopulmonary bypass and ultrafiltration. We initially decided to administer a fairly large bolus of citrulline immediately after initiation of cardiopulmonary bypass, with the goal of providing a 4-hour

CHD

TABLE 1. Model-dependent citrulline pharmacokinetic parameter estimates

	Patients 1 and 2	Patients 3 and 4	Patients 5-8
Dose (mg/kg)	50	100	150
R_{app} (μmol/[h · kg])	19.1 (15.9, 22.2)	14.7 (13.4, 16.0)	10.8 ± 1.9
k_{rem} (h^{-1})	0.78 (0.67, 0.89)	0.72 (0.87, 0.56)	0.68 ± 0.16
Volume of distribution (L/kg)	0.99 (0.97, 1.02)	0.99 (0.77, 1.22)	0.89 ± 0.24
Clearance (L/[h · kg])	0.78 (0.65, 0.91)	0.68 (0.67, 0.8)	0.58 ± 0.05

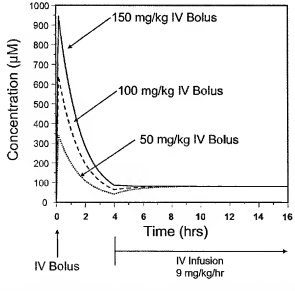
Data represent means of individual values (± SD for patients 5 through 8; individual values are given in parentheses for patients 1 and 2 and patients 3 and 4) of parameter estimates from pharmacokinetic model shown in Figure 2. R_{app}, Rate of citrulline appearance; k_{rem}, constant of citrulline removal.

target trough level of approximately 80 to 100 μmol/L. This target level was intentionally above the threshold value of approximately 40 μmol/L that we had previously identified as potentially protective against postoperative pulmonary hypertension in our studies with orally administered citrulline.[10] With an intravenously administered citrulline bolus of 150 mg/kg, we were able to achieve that target 4-hour trough level. An alternative approach would have been to use a smaller initial bolus followed by an immediate continuous infusion, but the mechanics of cardiopulmonary bypass and ultrafiltration both during and after bypass made this approach impractical.

In phase 1 of the study, we determined that the half-life of intravenously administered citrulline is fairly short at approximately 60 minutes. The volume of distribution of citrulline was estimated between 0.8 and 1.0 L/kg among the dosage groups in Phase 1 (Table 1). This value suggests that citrulline distributes to extravascular spaces. The high postdosing levels observed during phase 2 suggest that citrulline rapidly distributes out of the vascular space. The short half-life of citrulline is largely due to efficient elimination by the body, with clearance 0.6 to 0.8 L/(kg · h).

We did not study intravenously administered citrulline in patients who were not undergoing cardiac surgery. With computer modeling of data obtained from the second postoperative bolus dose in phase 1 of this study, however, we estimated that a bolus of 20 mg/kg immediately followed by a continuous infusion of 9 mg/(kg · h) would rapidly achieve steady state at plasma levels of 100 μmol/L in other patient groups at risk for pulmonary hypertension, in whom the effects of cardiopulmonary bypass would not be a concern in establishing the pharmacokinetics of citrulline. This noncardiac surgical protocol would need to be validated in further studies.

Determination of whether intravenously administered citrulline is a potential therapy for postoperative pulmonary hypertension will require a randomized clinical trial. We have recently initiated a trial of intravenously administered citrulline versus saline placebo according the described pro-



Bolus (mg/kg)	Model Predicted Peak (μM)	Model Predicted Trough (4hrs) (μM)
50	340	42
100	645	64
150	945	87

Figure 4. Pharmacokinetic modeling of intravenously administered *(IV)* bolus of citrulline given at beginning of surgery, followed 4 hours later by continuous infusion. Bolus dose of 150 mg/kg was determined most likely to yield 4-hour trough of 80 to 100 μmol/L, and infusion of 9 mg/(kg · h) was predicted to achieve steady state. This protocol was used in phase 2.

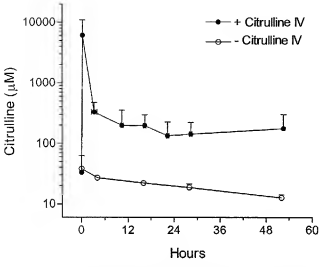


Figure 5. Plasma concentration–time profile of citrulline in phase 2. Patients 9 to 17 were administered citrulline 150 mg/kg intravenously *(IV)* in operating room on cardiopulmonary bypass, followed by continuous infusion at 9 mg/(kg · h) initiated 4 hours later after admission to pediatric intensive care unit. Target citrulline levels were achieved with this citrulline dosing regimen. For comparison, citrulline plasma levels in infants undergoing similar procedures without citrulline treatment in previously published study are shown.[11]

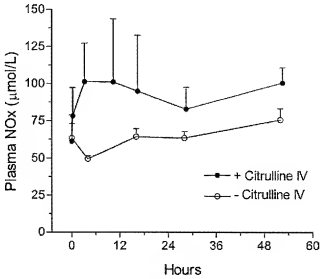


Figure 7. Plasma concentration–time profile of nitric oxide metabolites *(NOx)* in phase 2. Patients 9 to 17 were administered citrulline 150 mg/kg intravenously *(IV)* in operating room on cardiopulmonary bypass, followed by continuous infusion at 9 mg/(kg · h) initiated 4 hours later after admission to pediatric intensive care unit. For comparison, nitric oxide metabolite plasma levels in infants undergoing similar procedures without citrulline treatment in previously published study are shown.[19]

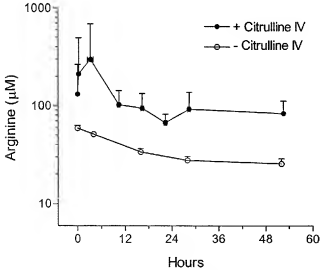


Figure 6. Plasma concentration–time profile of arginine in phase 2. Patients 9 to 17 were administered citrulline 150 mg/kg intravenously *(IV)* in operating room on cardiopulmonary bypass, followed by continuous infusion at 9 mg/(kg · h) initiated 4 hours later after admission to pediatric intensive care unit. For comparison, arginine plasma levels in infants undergoing similar procedures without citrulline treatment in previously published study are shown.[11]

tocol in infants and children undergoing these same five cardiac surgical procedures, with a goal of reducing the incidence of postoperative pulmonary hypertension by 50%. In addition, the efficacy of combination therapy of orally or intravenously administered citrulline and other therapies for pulmonary hypertension, such as inhaled nitric oxide, nebulized inhaled epoprostenol, and orally administered sildenafil citrate, should be investigated.

References

1. Steinhorn RH, Fineman JR. The pathophysiology of pulmonary hypertension in congenital heart disease. *Artif Organs.* 1999;23:970-4.
2. Bandla HP, Hopkins RL, Beckerman RC, Gozal D. Pulmonary risk factors compromising postoperative recovery after surgical repair for congenital heart disease. *Chest.* 1999;116:740-7.
3. Gentles TL, Mayer JE Jr, Gauvreau K, Newburger JW, Lock JE, Kupferschmid JP, et al. Fontan operation in five hundred consecutive patients: factors influencing early and late outcome. *J Cardiovasc Surg.* 1997;114:376-91.
4. Nakajima Y, Momma K, Seguchi M, Nakazawa M, Imai Y. Pulmonary hypertension in patients with complete transposition of the great arteries: midterm results after surgery. *Pediatr Cardiol.* 1996;17: 104-7.
5. Schulze-Neick I, Li J, Punny DJ, Reington AN. Pulmonary vascular resistance after cardiopulmonary bypass in infants: effect on postoperative recovery. *J Thorac Cardiovasc Surg.* 2001;121:1033-9.
6. Russell IA, Zwass M, Fineman JR, Balea M, Rouine-Rapp K, Brook M, et al. The effects of inhaled nitric oxide on postoperative pulmo-

nary hypertension in infants and children undergoing surgical repair of congenital heart disease. *Anesth Analg.* 1998;87:46-51.
7. Zobel G, Gamillscheg A, Schwinger W, Berger J, Urlesberger B, Dacar D, et al. Inhaled nitric oxide in infants and children after open heart surgery. *J Cardiovasc Surg.* 1998;39:79-86.
8. Moncada S, Higgs A. The L-arginine–nitric oxide pathway. *N Engl J Med.* 1993;329:2002-12.
9. Pearson DL, Dawling S, Walsh WF, Haines JL, Christman BW, Bazyk A, et al. Neonatal pulmonary hypertension—urea-cycle intermediates, nitric oxide production, and carbamoyl-phosphate synthetase function. *N Engl J Med.* 2001;344:1832-8.
10. Barr FE, Beverley H, VanHook K, Cermak E, Christian K, Drinkwater D, et al. Effect of cardiopulmonary bypass on urea cycle intermediates and nitric oxide levels after congenital heart surge. *J Pediatr.* 2003;142:26-30.
11. Smith HA, Canter JA, Christian KG, Drinkwater DC, Scholl FG, Christman BW, et al. Nitric oxide precursors and congenital heart surgery: a randomized controlled trial of oral citrulline. *J Thorac Cardiovasc Surg.* 2006;132:58-65.

Madhumita Ananthakrishnan, Frederick E. Barr, Marshall L. Summar, Heidi A. Smith, Mark Kaplowitz, Gary Cunningham, Jordan Magarik, Yongmei Zhang and Candice D. Fike

Am J Physiol Lung Cell Mol Physiol 297:506-511, 2009. First published Jul 17, 2009; doi:10.1152/ajplung.00017.2009

Am J Physiol Lung Cell Mol Physiol 297: L506–L511, 2009.
First published July 17, 2009; doi:10.1152/ajplung.00017.2009.

Exhibit C

L-Citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets

Madhumita Ananthakrishnan, Frederick E. Barr, Marshall L. Summar, Heidi A. Smith, Mark Kaplowitz, Gary Cunningham, Jordan Magarik, Yongmei Zhang, and Candice D. Fike

Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee

Submitted 21 January 2009; accepted in final form 9 July 2009

Ananthakrishnan M, Barr FE, Summar ML, Smith HA, Kaplowitz M, Cunningham G, Magarik J, Zhang Y, Fike CD. L-Citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets. *Am J Physiol Lung Cell Mol Physiol* 297: L506–L511, 2009. First published July 17, 2009; doi:10.1152/ajplung.00017.2009.— Newborn piglets develop pulmonary hypertension and have diminished pulmonary vascular nitric oxide (NO) production when exposed to chronic hypoxia. NO is produced by endothelial NO synthase (eNOS) in the pulmonary vascular endothelium using L-arginine as a substrate and producing L-citrulline as a byproduct. L-Citrulline is metabolized to L-arginine by two enzymes that are colocated with eNOS in pulmonary vascular endothelial cells. The purpose of this study was to determine whether oral supplementation with L-citrulline during exposure of newborn piglets to 10 days of chronic hypoxia would prevent the development of pulmonary hypertension and increase pulmonary NO production. A total of 17 hypoxic and 17 normoxic control piglets were studied. Six of the 17 hypoxic piglets were supplemented with oral L-citrulline starting on the first day of hypoxia. L-Citrulline supplementation was provided orally twice a day. After 10 days of hypoxia or normoxia, the animals were anesthetized, hemodynamic measurements were performed, and the lungs were perfused in situ. Pulmonary arterial pressure and pulmonary vascular resistance were significantly lower in hypoxic animals treated with L-citrulline compared with untreated hypoxic animals ($P < 0.001$). In vivo exhaled NO production ($P = 0.03$) and nitrite/nitrate accumulation in the perfusate of isolated lungs ($P = 0.04$) were significantly higher in L-citrulline-treated hypoxic animals compared with untreated hypoxic animals. L-Citrulline supplementation ameliorated the development of pulmonary hypertension and increased NO production in piglets exposed to chronic hypoxia. We speculate that L-citrulline may benefit neonates exposed to prolonged periods of hypoxia from cardiac or pulmonary causes.

nitric oxide synthase; nitric oxide; L-arginine recycling

INFANTS WITH CHRONIC LUNG DISEASE and cyanotic congenital heart disease frequently suffer from hypoxia. Because of its effects on both existing and developing pulmonary arteries, chronic hypoxia causes progressive changes in both the function and structure of the pulmonary circulation (28, 31). Ultimately, chronic hypoxia results in severe pulmonary hypertension culminating in right-sided heart failure and death. Currently, the therapy for pulmonary hypertension in infants suffering from chronic cardiopulmonary disorders associated with persistent or episodic hypoxia is largely limited to improving the underlying cardiopulmonary disorder and attempts to achieve adequate oxygenation (1, 2, 23, 31). The need for novel therapies to treat infants with chronic progressive neonatal pulmonary hypertension is well acknowledged (1–3, 14, 23).

Address for reprint requests and other correspondence: C. D. Fike, Division of Neonatology/Research, 2215 B Garland Ave., 1125 MRB IV/Light Hall, Nashville, TN 37232-0656 (e-mail: candice.fike@vanderbilt.edu).

The piglet is an excellent species for the study of neonatal pulmonary hypertension since adaptation of the pulmonary circulation to extra-uterine life is similar in pigs and humans (14). Changes in pulmonary blood vessels found in piglets exposed to hypoxia approximate those found in human infants with pulmonary hypertension (15). We have previously shown that newborn piglets develop pulmonary hypertension when exposed to chronic hypoxia (9). Moreover, we have shown that the development of pulmonary hypertension in piglets exposed to 10 days of chronic hypoxia is associated with impaired production of the vasodilator nitric oxide (NO) (14).

NO is produced by endothelial NO synthase (eNOS) in the pulmonary vascular endothelium using L-arginine as a substrate and producing L-citrulline as a by-product. In turn, L-arginine can be synthesized from L-citrulline, providing a recycling pathway for the conversion of L-citrulline to NO via L-arginine (30). Plasmalemmal caveolae, the site of the L-citrulline-to-L-arginine recycling pathway, may be the principal source of L-arginine available to eNOS (12, 13, 30). Via this recycling pathway, the availability of L-citrulline may regulate NO production by eNOS in the pulmonary circulation.

The purpose of this study was to determine whether oral supplementation with L-citrulline during exposure of newborn piglets to 10 days of chronic hypoxia would prevent the development of pulmonary hypertension and the concomitant reduction in NO production.

METHODS

Animal care. All experimental protocols were performed in adherence with the National Institutes of Health guidelines for the use of experimental animals and approved by the Animal Care and Use Committee of Vanderbilt University Medical Center. The animal resource facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. A total of 17 hypoxic and 17 normoxic control piglets were studied. Normoxic control animals were studied on the day of arrival from the farm at 12 days of age. The hypoxic pigs (2 days old) were placed in a normobaric hypoxic chamber for 10–11 days. Normobaric hypoxia was provided using compressed air and nitrogen to create inspired oxygen of 8–11% (Po_2 of 60–72 Torr) and Co_2 was maintained at 3–6 Torr by absorption with soda lime. The animals were monitored with daily weights and physical exam twice daily. They were fed ad libitum with sow milk replacer from a feeding device in the cage.

L-Citrulline supplementation. Six of the 17 hypoxic piglets were supplemented with oral L-citrulline starting on the first day of the hypoxic exposure. L-Citrulline supplementation was provided at a dose of 0.13 g/kg body wt twice a day using a syringe to deliver the dose orally. If it appeared to study personnel that the piglet had not ingested the majority of a dose, it was repeated. L-Citrulline was mixed using a preparation (Sigma Pharmaccuticals, 98% purity) at a concentration of 0.13 g/ml of distilled water. When completely dissolved, this solution was passed through a 0.20-μm filter.

In vivo hemodynamics. In vivo hemodynamics were measured in six of the normoxic control piglets and in all of the hypoxic piglets. For these measurements, the animals were weighed and then preanesthetized with Ketamine (15 mg/kg) and Acepromazine (2 mg/kg) intramuscularly. A tracheostomy, venous and arterial catheters, and thermistor were then placed as previously described using intravenous pentobarbital for sedation (10). Pulmonary artery pressure, left ventricular end diastolic pressure, and cardiac output were measured. Cardiac output was measured by a thermodilution technique (model 9520 thermodilution cardiac output computer, Edwards Laboratory, Irvine, CA) using a thermistor in the aortic arch and the left ventricle catheter as an injection port. Cardiac output was measured at end expiration as the mean of three injections of 3 ml of normal saline (0°C). Exhaled NO was measured as described below. During the in vivo measurements, animals were ventilated with room air using a piston-type ventilator at a tidal volume of 15–20 ml/kg, end-expiratory pressure of 2 Torr, and a respiratory rate of 15–20 breaths/min. Hemodynamic measurements were obtained in all hypoxic animals and six control animals. In our past experience as in this study, it is not always possible to obtain in vivo hemodynamic data on every animal for technical reasons. The most common difficulty encountered is the inability and length of time needed to place and advance a right heart catheter into the pulmonary artery to measure pulmonary artery pressure. Because of this difficulty, we did not attempt to obtain hemodynamic data in all control animals.

Exhaled NO measurement. For exhaled NO measurement in anesthetized animals, expiratory gas was sampled two to three times for 3-min periods each and passed through a chemiluminescence analyzer (model 270B NOA; Sievers, Boulder, CO) to measure NO concentration as previously described (11). Exhaled NO production (nmol/min) was calculated using minute ventilation and the measured exhaled NO concentration.

Isolated lung perfusions. All control and hypoxic animals used for hemodynamic measurements and an additional 11 control piglets were used in isolated lung perfusions. The lungs were isolated and perfused in situ with a Krebs Ringer bicarbonate (KRB) solution containing 5% dextran, molecular weight of 70,000, at 37°C and ventilated with a normoxic gas mixture (21% O_2 and 5% CO_2) as previously described (10). The lungs were perfused for 30–60 min until a stable pulmonary arterial pressure was achieved. Perfusate samples (1 ml) were then removed from the left atrial cannula every 10 min for a 60-min period. The perfusate samples were centrifuged, and the supernatant was stored at −80°C for future analysis of nitrite/nitrate (NOx^-) concentrations as described below. At the end of the perfusion, the volume of perfusate remaining in the circuit and reservoir was measured. In some cases, lung tissue was collected immediately following the perfusion, frozen with liquid nitrogen, and then stored at −80°C for later measurement of eNOS and nNOS content as described below. Isolated lung perfusions were attempted in all animals. It is our experience, as in this study, that it is not possible to successfully isolate and perfuse lungs in all animals for technical reasons.

NOx^- measurement. A chemiluminescence analysis described previously was used to determine perfusate NOx^- concentration (nmol/ml) at each collection time. (10, 34) Perfusate (20 μl) was injected into the reaction chamber of a chemiluminescence NO analyzer (model 170B NOA, Sievers). The reaction chamber contained vanadium (III) chloride in 1 M HCl heated to 90°C to reduce nitrite and nitrate to NO gas. The NO gas was carried into the analyzer using a constant flow of N_2 gas via a gas bubble trap containing 1 M NaOH to remove HCl vapor. A standard curve was generated by adding known amounts of $NaNO_3$ to distilled water and assaying as described for the perfusion samples.

The perfusate NOx^- concentration (nmol/ml) was calculated for each collection time by multiplying the perfusate concentration of NOx^- at that sample collection time by the volume of the system (perfusion circuit + reservoir) at the sample collection time plus the amount of NOx^- removed with all previous samples. The rate of NOx^- production was determined from the slope of a linear regression line fit to the amount of NOx^- in the perfusate vs. time for the first 60 min of the collection period.

Plasma amino acid measurements. On the day of hemodynamic measurements and/or lung perfusion study, for normoxic control and both L-citrulline-treated and -untreated chronic hypoxic animals, blood was drawn before the study was started and the plasma frozen at −80°C for later determination of amino acid levels. For the L-citrulline-treated hypoxic animals, a blood sample was obtained 12 h after the last dose of citrulline to measure the trough level of this amino acid. We wanted to verify that L-citrulline levels in treated animals were greater than those in untreated animals. Therefore, in some of the L-citrulline-treated animals ($n = 3$), after blood sampling for a trough level, a dose of L-citrulline was given via nasogastric tube. Following this dose, blood samples were drawn every 30 min for 90 min (the length of the in vivo studies). All samples were spun, and the plasma was collected and frozen at −80°C for amino acid analysis.

Concentrations of plasma citrulline and arginine were determined by amino-acid analysis on protein-free extracts. Amino acids were separated by cation-exchange chromatography using a Hitachi L8800 amino acid analyzer (Hitachi USA, San Jose, CA). Calibration of the analyzer was performed before piglet samples were tested.

Western blot of eNOS and nNOS in lung tissue. Using a standard immunoblot technique as previously described, we analyzed samples of whole lung homogenates from normoxic controls ($n = 3$) and untreated hypoxic ($n = 3$) and L-citrulline-treated hypoxic ($n = 3$) animals for eNOS and nNOS. We used 10 and 30 mg of total protein for eNOS and nNOS, respectively, a dilution of primary eNOS or nNOS antibody of 1:500 (BD transduction), and a dilution of secondary anti-mouse antibody conjugated to horseradish peroxidase of 1:5,000 (11).

Calculations and statistics. Pulmonary vascular resistance was calculated from the in vivo hemodynamic measurements: (pulmonary arterial pressure − left ventricular end diastolic pressure) ÷ (cardiac output/body wt).

Data are presented as means ± SD. The one-way ANOVA with Fisher's protected least significant difference (PLSD) post hoc comparison test was used to compare data between normoxic control and untreated hypoxic and L-citrulline-treated hypoxic animals. A P value of <0.05 was considered significant (21).

RESULTS

In vivo hemodynamic measurements. Both L-citrulline-treated and -untreated chronic hypoxic animals had lower cardiac output and weights and higher left ventricular end-diastolic pressure measurements on the day of study at 12–13 days of age than comparable age normoxic control piglets (Table 1). We have previously shown that piglets grown under hypoxic conditions have less weight gain than those grown under normoxic conditions (9). Measurements of aortic pressure and arterial Po_2 (Pa_{O_2}) were similar (Pa_{O_2} was 74 ± 13 Torr in normoxic control piglets, 74 ± 16 Torr in untreated hypoxic piglets, and 78 ± 16 Torr in L-citrulline-treated hypoxic piglets) among groups. Values for arterial Pco_2 (Pa_{CO_2}) were significantly lower ($P = 0.04$) in the L-citrulline-treated hypoxic animals (30 ± 3 Torr) compared with both normoxic controls (39 ± 6 Torr) and untreated hypoxic (41 ± 12 Torr) animals. However, since the values of pH did not differ significantly between any of the groups of animals (Table 1), these differences in Pa_{CO_2} are unlikely to have had any physiological impact on the hemodynamic measurements.

Notably, as shown in Fig. 1A, L-citrulline-treated hypoxic animals had significantly lower pulmonary artery pressures

Table 1. *Data for normoxic control, chronically hypoxic, and L-citrulline-treated chronically hypoxic piglets*

Treatment Group	Weight at 12 Days of Age, kg	Aortic Pressure, cmH_2O	LVEDP, cmH_2O	Cardiac Output, $ml \cdot min^{-1} \cdot kg^{-1}$	Arterial pH
Controls (n = 6)	3.94±0.7	91±9	5.2±1.5	414±105	7.38±0.12
Chronic hypoxic (n = 11)	2.76±0.5*	100±12	7.4±1.7*	244±00*	7.38±0.04
Citrulline hypoxic (n = 6)	2.6±0.23*	97±15	7.2±1.1*	270±71*	7.36±0.05

Values are means ± SD. LVEDP, left ventricular end-diastolic pressure. *Significnat difference vs. normoxic controls ($P < 0.05$; ANOVA with post hoc comparison test).

than untreated hypoxic animals. In addition, as shown in Fig. 1*B*, calculated pulmonary vascular resistance in those hypoxic animals treated with L-citrulline were significantly lower than those of untreated hypoxic animals. Furthermore, pulmonary vascular resistances were similar in L-citrulline-treated hypoxic animals and normoxic controls.

Exhaled NO output and perfusate NOx^-. As shown in Fig. 2*A*, exhaled NO output in normoxic controls and L-citrulline-treated hypoxic animals were higher than exhaled NO output in untreated hypoxic animals. However, exhaled NO output did not differ between normoxic control and L-citrulline-treated hypoxic animals.

As shown in Fig. 2*B*, lungs from both the normoxic control and L-citrulline-treated hypoxic animals had significantly higher NOx^- accumulation rates than lungs from untreated hypoxic animals. Furthermore, there was no difference in the rate of NOx^- accumulation between lungs from L-citrulline-treated hypoxic animals and normoxic controls.

Plasma amino acids. As shown in Table 2, although not reaching statistical significance, plasma L-citrulline levels in untreated chronic hypoxic piglets were less than trough L-citrulline levels in treated hypoxic piglets. Moreover, when drawn 90 min after a dose, levels of L-citrulline in treated hypoxic animals were almost twice that of the untreated

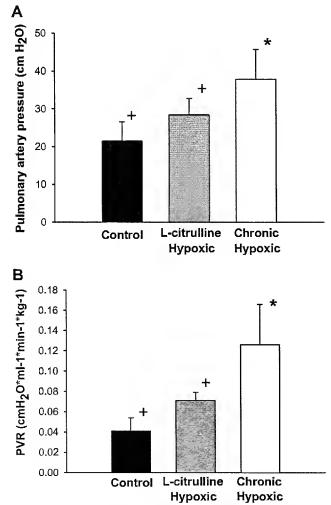


Fig. 1. *A*: mean pulmonary arterial pressure measurements in normoxic control (n = 6), chronically hypoxic (n = 11), and L-citrulline-treated chronically hypoxic (n = 6) piglets. *B*: calculated pulmonary vascular resistance in normoxic control (n = 6), chronically hypoxic (n = 11), and L-citrulline-treated chronically hypoxic (n = 6) piglets. Values are means ± SD. Significantly different from normoxic control (*) and chronically hypoxic (+) ($P < 0.05$; ANOVA with post hoc comparison test).

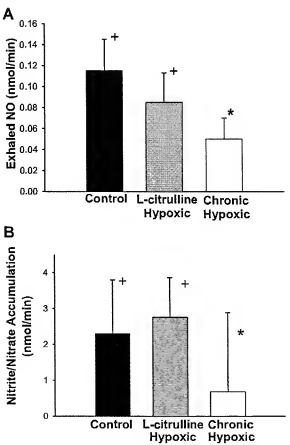


Fig. 2. *A*: exhaled nitric oxide in normoxic control (n = 6), chronically hypoxic (n = 11), and L-citrulline-treated chronically hypoxic (n = 5) piglets. *B*: nitrite/nitrate accumulation in lung perfusate in normoxic control (n = 17), chronically hypoxic (n = 9), and L-citrulline-treated chronically hypoxic (n = 5) piglets. Values are means ± SD. Significantly different from normoxic control (*) and chronically hypoxic (+) ($P < 0.05$; ANOVA with post hoc comparison test).

Table 2. *Plasma amino acid levels for normoxic control, chronically hypoxic, and L-citrulline-treated chronically hypoxic piglets*

Treatment Group	Citrulline, μM	Arginine, μM
Normoxic controls (n =10)	71±20	112±49
Chronic Hypoxic (n =8)	111±67	51±31*
L-Citrulline-treated hypoxic (90 min; n =3)	219±63*†	43±8*
L-Citrulline-treated hypoxic (trough; n = 6)	161±13*	39±24*

Values are means ± SD. Trough, plasma level ~12 h after L-citrulline dose; 90 min, plasma level 90 min after administration of L-citrulline dose. *Significant difference vs. normoxic controls ($P < 0.05$; ANOVA with post hoc comparison test). †Significant difference vs. untreated chronic hypoxics ($P < 0.05$; ANOVA with post hoc comparison test).

chronic hypoxic animals. Levels of L-citrulline obtained at 30 (135 ± 60 μM) and 60 (156 ± 9 μM) min after a dose did not differ significantly from the 90-min value. Regardless of the time the sample was drawn, plasma arginine levels were not higher in L-citrulline-treated chronic hypoxic animals compared with untreated hypoxic animals.

Western blot for lung eNOS and nNOS protein. As shown in Fig. 3 and consistent with our previous studies (11), the amount of eNOS protein present in the lung tissue of normoxic control animals was significantly higher than that present in the lungs of untreated hypoxic animals. Furthermore, the amount of eNOS protein present in the lung tissue of L-citrulline-treated hypoxic piglets was not significantly different from that in the untreated hypoxic animals and was significantly lower than eNOS protein levels in normoxic control animals. As shown in Fig. 4, there was no difference in nNOS protein levels among the three groups.

DISCUSSION

In this study, we found that L-citrulline supplementation ameliorates the development of pulmonary hypertension in newborn piglets exposed to 10 days of chronic hypoxia. To our knowledge, this is the first study showing the effectiveness of L-citrulline in preventing the development of pulmonary hypertension in either newborn or more mature animal models of this disease.

Other important findings in this study are that both exhaled NO production and pulmonary vascular NOx^- accumulation rates are greater in L-citrulline-treated hypoxic piglets than in untreated hypoxic piglets. Thus our findings clearly show that L-citrulline supplementation significantly increased pulmonary NO production. In addition, our finding that the amounts of eNOS and nNOS protein are unchanged in the L-citrulline-treated hypoxic animals suggests that the mechanism for this increase in pulmonary NO production is not an increase in NOS expression.

Based on the current literature (13, 17 ,30 ,32), the mechanism by which L-citrulline mediates an increase in NO production could be by improving NOS function. One possible mechanism for improving NOS function is by increasing the amount of L-arginine available as a substrate for eNOS. Assessment of arginine availability for NO synthesis has been a challenge that has been addressed by many investigators. Plasma levels of arginine in the L-citrulline-treated animals in this study were not significantly increased compared with untreated hypoxic animals. However, this finding was not surprising since total cellular levels of L-arginine have not been

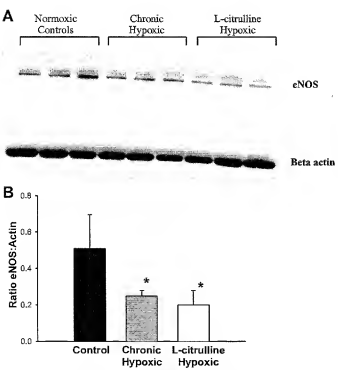


Fig. 3. *A*: immunoblot for eNOS protein reprobed for beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets. *B*: densitometry of eNOS normalized to beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets Values are means ± SD. *Significantly different from normoxic control ($P < 0.05$; ANOVA with post hoc comparison test).

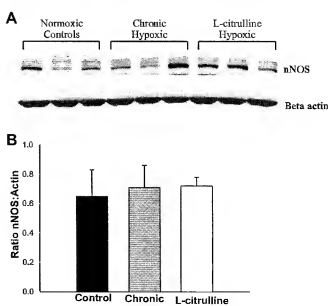


Fig. 4. *A*: immunoblot for nNOS protein reprobed for beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets. *B*: densitometry of nNOS normalized to beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets. Values are means ± SD.

found to accurately reflect subcellular levels of L-arginine available for NO synthesis. Su and Block (32) attempted to show that decreased NO production in pulmonary endothelial cells exposed to hypoxia was due to a decrease in cellular L-arginine content. They found that, rather than being decreased, cellular L-arginine content was actually increased by degradation of cellular proteins in response to hypoxia and hypothesized that this increased supply of L-arginine was unavailable to eNOS (32). Solomonson et al. in 2003 showed that providing L-arginine to endothelial cells increased NO production only slightly compared with the more dramatic increase in endothelial NO production found with L-citrulline supplementation (30). In addition, L-citrulline supplementation increased total cellular arginine only slightly compared with the significant increase in total cellular arginine after L-arginine supplementation. Thus, similar to Su and Block, these authors concluded that there was no correlation between total cellular arginine and endothelial NO production (30). Based on findings from these and other studies (13, 17), eNOS function seems to be dependent on a pool of arginine that is isolated from the bulk of intracellular arginine and is maintained through an efficient arginine regeneration enzymatic process in close proximity to eNOS.

This discordance between intracellular arginine and NO production, termed the "arginine paradox," explains the increase in NO production in the face of unchanged plasma arginine levels seen with L-citrulline supplementation in this study. L-Citrulline is a urea cycle intermediate metabolized to arginine by a recycling pathway consisting of two enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL). These two enzymes, AS and AL, have been found colocated with eNOS in pulmonary endothelial cells (7). It is thought that together these enzymes produce a separate subcellular pool of arginine used exclusively for NO synthesis. Tissue and plasma arginine levels cannot accurately measure this subcellular pool.

L-Citrulline may also have improved NO production and eNOS function by additional mechanisms. Recently, it has been suggested that, in the setting of ischemia and reperfusion injury, the enzyme eNOS (a dimer) uncouples and produces superoxide instead of NO (7). There is evidence that this uncoupling of eNOS occurs in the presence of low levels of arginine or BH4, a necessary cofactor for the production of NO (35). Hence, another potential action of L-citrulline in this study is the prevention of the uncoupling of eNOS by maintaining adequate levels of its substrate arginine. We have yet to explore this possibility.

L-Citrulline has been used in several patient populations with some success. In addition to those patients with urea cycle defects, patients with sickle cell disease receiving citrulline have shown improved disease symptoms (36). In children undergoing cardiopulmonary bypass at risk for development of postoperative pulmonary hypertension, Smith et al. recently showed that oral supplementation with L-citrulline increased both plasma citrulline and arginine levels (29). Moreover, postoperative pulmonary hypertension did not develop in those children who had plasma citrulline levels greater than 37 μM/l. Furthermore, intravenous L-citrulline has been shown to be safe and well tolerated in this same patient population of children undergoing bypass by Barr et al. (4).

Notably, L-citrulline therapy has been used in animal models of vascular diseases other than our model of chronic hypoxia-induced pulmonary hypertension. In rabbits fed a high-cholesterol diet, L-citrulline supplementation causes regression of atheromatous lesions (16). In spontaneously hypertensive rats, maternal supplementation with L-citrulline increased renal NO production and ameliorated hypertension in offspring (18). Therefore, it would seem that L-citrulline may be useful for improving NO dysfunction in conditions other than hypoxia-induced pulmonary hypertension.

Although L-citrulline has not been widely studied as a therapy for pulmonary hypertension, L-arginine supplementation has been used frequently with mixed results. For example, treatment with L-arginine has been shown to prevent the development of pulmonary hypertension in two adult rat models of pulmonary hypertension (22, 25). Furthermore, administration of L-arginine was shown to reverse evidence of postoperative pulmonary vascular endothelial dysfunction in children who had undergone cardiopulmonary bypass and to restore impaired pulmonary vasorelaxation in adults with pulmonary hypertension (6, 8, 20, 24, 27). Although these studies provide evidence that L-arginine may help prevent the development of pulmonary hypertension and may be helpful once pulmonary hypertension has developed, serious adverse effects of L-arginine treatment have been suggested, and variable results from L-arginine treatment have been reported (5, 26). Because arginine is involved in other processes in the body and is quickly metabolized by arginases in many cellular compartments, supplementation often requires high doses, i.e., 9 g/day, in adults (26). These massive doses are sometimes poorly tolerated, and patient compliance can be difficult to maintain (33).

There are several limitations of this study that merit comment. First, we have been unable to detect iNOS protein in lung tissue from newborn piglets using those antibodies currently commercially available. Thus, although we have shown that eNOS and nNOS protein levels in lung tissue are unchanged with L-citrulline therapy, we cannot rule out the possibility that an increase in iNOS protein contributes to the increase in NO production and decrease in pulmonary vascular resistance in L-citrulline-treated hypoxic piglets. In addition, eNOS has been shown to be present in respiratory epithelium as well as pulmonary vascular endothelium (34). Therefore, Western blots of whole lung homogenates cannot establish the precise anatomical site of any change in lung eNOS expression.

Another study limitation is that we did not measure AS and AL amounts or activities. It is possible that changes in the amount or activity of these enzymes that are colocated with eNOS could contribute to alterations in NO production. Yet another limitation is that our study findings do not address the possibility that L-citrulline therapy may have effects in normoxic animals. Also, because isolated lung perfusion requires disruption of the right ventricle morphology and can cause edema and distortion of the pulmonary architecture, we were unable to assess the effect of L-citrulline therapy on either right ventricular hypertrophy or pulmonary vascular remodeling. We were unable to assess the changes in pulmonary vasoreactivity since the agonists used to determine reactivity can potentially alter lung NO production. In addition, vessels harvested from isolated perfused lung preparations are no longer viable for use in pressurized, cannulated artery studies.

Further studies are required to more extensively evaluate the mechanisms underlying the effect of L-citrulline therapy on NOS function, potential changes in vasoreactivity, and the development of pulmonary hypertension.

In summary, our findings show that L-citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets. We also provide evidence that the effectiveness of citrulline is due to increased NO production, which is likely due at least in part to an increase in NOS function since neither eNOS nor nNOS protein levels are changed. It is possible that L-citrulline may be a useful therapy in neonates at risk of developing pulmonary hypertension due to conditions associated with impaired NO function, including chronic or intermittent unresolved hypoxia.

ACKNOWLEDGMENTS

This work was supported by an American Heart Association affiliate grant to C. D. Fike.

REFERENCES

1. **Aiman SH.** Monitoring cardiovascular function in infants with chronic lung disease of prematurity. *Arch Dis Child Fetal Neonatal Ed* 87: F15–F18, 2002.
2. **Allen J, subcommittee AoP ATS.** Statement on the care of the child with chronic lung disease of infancy and childhood. *Am J Respir Crit Care Med* 168: 356–396, 2003.
3. **Aschner J.** New therapies for pulmonary hypertension in neonates and children. *Pediatric Pulmonol* 26S: S132–S135, 2004.
4. **Barr FE, Tirona RG, Taylor MB, Rice G, Arnold J, Cunningham G, Smith HA, Campbell A, Canter JA, Christian KG, Drinkwater DC, Scholl F, Kavanaugh-McHugh A, Summar ML.** Pharmacokinetics and safety of intravenously administered citrulline in children undergoing congenital heart surgery:potential therapy for postoperative pulmonary hypertension. *J Thorac Cardiovasc Surg* 134: 319–326, 2007.
5. **Boger RH.** L-Arginine therapy in cardiovascular pathologies: beneficial or dangerous? *Curr Opin Clin Nutr and Met Care* 11: 55–61, 2008.
6. **Boger RH, Bode-Boger SM, Heinzel D.** Differential systemic and pulmonary haemodynamic effects of L-arginine in patients with coronary heart disease and primary pulmonary hypertension. *Int Clin Pharmacol Ther* 34: 323–328, 1996.
7. **Dragoni S, Gori Stolfo GD T, Sicuro S, Forconi Parker JD S.** Folic acid does not limit endothelial dysfunction induced by ischemia and reperfusion. *J Cardiovasc Pharmacol* 46: 494–497, 2005.
8. **Drexler H, Zeiher AM, Meinzer K, Just H.** Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* 338: 1546–1550, 1991.
9. **Fike CD, Kaplowitz MR.** Effect of chronic hypoxia on pulmonary vascular pressures in isolated lungs of newborn pigs. *J Appl Physiol* 77: 2853–2862, 1994.
10. **Fike CD, Kaplowitz MR, Rehorst-Paea LA, Nelin LD.** L-Arginine increases nitric oxide production in isolated lungs of chronically hypoxic newborn pigs. *J Appl Physiol* 88: 1797–1803, 2000.
11. **Fike CD, Kaplowitz MR, Thomas CJ, Nelin LD.** Chronic hypoxia decreases nitric oxide production and endothelial nitric oxide synthase in newborn pig lungs. *Am J Physiol Lung Cell Mol Physiol* 274: L517–L526, 1998.
12. **Flam BR, Hartmann PJ, Harrell-Booth M, Solomonson LP, Eichler DC.** Cavcolar localization of arginine regencration enzymes, arginiosuccinate synthase, and lyase, with endothelial nitric oxide synthase. *Nitric Oxide* 5: 187–197, 2001.
13. **Goodwin BL, Solomonson LP, Eichler DC.** Argininosuccinate synthase expression is required to maintain nitric oxide production and cell viability in aortic endothelial cells. *J Biol Chem* 279: 18353–18360, 2004.
14. **Haworth SG, Hislop AA.** Adaptation of the pulmonary circulation to extra-uterine life in the pig and its relevance to the human infant. *Cardiovasc Res* 15: 108–119, 1981.
15. **Haworth SG, Hislop AA.** Effect of hypoxia on adaptation of the pulmonary circulation to extra-uterine life in the pig. *Cardiovasc Res* 16: 293–303, 1982.
16. **Hayashi T, Juliet PAR, Matsui-Hirai H, Miyazaki A, Fukatsu A, Funami J, Iguchi A, Ignarro LJ.** L-Citrulline and L-arginine supplementation retards the progression of high-cholesterol-diet-induced atherosclerosis in rabbits. *Proc Natl Acad Sci USA* 102: 13681–13686, 2005.
17. **Hecker Sessa WC M, Harris HJ, Anggard EE, Vane JR.** The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci USA* 87: 8612–8616, 1990.
18. **Koeners MP, Van Faassen EE, Wesseling S, Sain-van der Velden M, Koomans HA, Braam B, Joles JA.** Maternal supplementation with citrulline increases renal nitric oxide in young spontaneously hypertensive rats and has long-term antihypertensive effects. *Hypertension* 50: 1077–1084, 2007.
19. **Liu JQ, Zelko IN, Erbynn EM, Sham JSK, Folz RJ.** Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase ($gp91^{phox}$). *Am J Physiol Lung Cell Mol Physiol* 290: L2–L10, 2006.
20. **Mehta S, Stewart DJ, Langleben D, Levy RD.** Short-term pulmonary vasodilation with L-arginine in pulmonary hypertension. *Circulation* 92: 1539–1545, 1995.
21. **Meier U.** A note on the power of Fisher's least significant difference procedure. *Pharm Stat* 5: 253–263, 2006.
22. **Mitani Y, Maruyama K, Minoru S.** Prolonged administration of L-arginine ameliorates chronic pulmonary hypertension and pulmonary vascular remodeling in rats. *Circulation* 96: 689–697, 1997.
23. **Mupanemunda RH.** Current status of inhaled nitric oxide therapy in the perinatal period. *Early Hum Dev* 47: 247–262, 1997.
24. **Pernow J, Bohm F, Beltran E, Gonon A.** L-Arginine protects from ischemia-reperfusion-induced endothelial dysfunction in humans in vivo. *J Appl Physiol* 95: 2218–2222, 2003.
25. **Sasaki S, Asano M, Ukai T, Nomura N, Maruyama K, Manabe T, Mishima A.** Nitric oxide formation and plasma L-arginine levels in pulmonary hypertensive rats. *Respir Med* 98: 205–212, 2004.
26. **Schulman SP, Becker LC, Kass DA, Champion HC, Terrin ML, Forman S, Ernst KV, Kelemen MD, Townsend SN, Capriotti A, Hare JM, Gerstenblith G.** L-Arginine therapy in acute myocardial infarction: the vascular interaction with age in myocardial infarction (VINTAGE MI) randomized clinical trial. *JAMA* 295: 58–64, 2006.
27. **Schulze-Neick I, Penny DJ, Rigby ML, Morgan C, Kelleher A, Collins P, Li J,Bush A, Shinebourne EA, Redington AN.** L-Arginine and substance P reverse the pulmonary endothelial dysfunction caused by congenital heart surgery. *Circulation* 100: 749–755, 1999.
28. **Shimoda L, Sham JSK, Sylvester JT.** Altered pulmonary vasoreactivity in the chronically hypoxic lung. *Physiol Res* 49: 549–560. 2000.
29. **Smith HAB, Canter JA, Christian KG, Drinkwater DC, Scholl FG, Christman BW, Rice GD, Barr FE, Summar ML.** Nitric oxide precursors and congenital heart surgery: a randomized controlled trial of oral citrulline. *J Thorac Cardiovasc Surg* 132: 58–65, 2006.
30. **Solomonson LP, Flam BR, Pendleton LC, Goodwin BL, Eichler DC.** The caveolar nitric oxide synthase/arginine regeneration system for NO production in endothelial cells. *J Exp Biol* 206: 2083–2087, 2003.
31. **Subhedar NV.** Recent advances in diagnosis and management of pulmonary hypertension in chronic lung disease. *Acta Paediatr Suppl* 444: 29–32, 2004.
32. **Su Y, Block ER.** Acute hypoxia increases intracellular L-arginine content in cultured porcine pulmonary artery endothelial cells. *J Cell Physiol* 167: 349–353, 1996.
33. **Tenenbaum A, Fisman EZ, Motro M.** L-Arginine: rediscovery in progress. *Cardiology* 90: 153–159, 1998.
34. **Turley JE, Nelin LD, Kaplowitz MR, Zhang Y, Fike CD.** Exhaled NO is reduced at an early stage of hypoxia-induced pulmonary hypertension in newborn piglets. *Am J Physiol Lung Cell Mol Physiol* 284: L489–L500, 2003.
35. **Verma S, Maitland A, Weisel RD, Fedak PWM, Pomroy NC, Li SH, Mickle DAG, Li RK, Rao V.** Novel cardioprotective effects of tetrahydrobiopterin after anoxia and reoxygenation: identifying cellular targets for pharmacologic manipulation. *J Thorac Cardiovasc Surg* 123: 1074–1083, 2002.
36. **Waugh WH, Daeschner CWIII, Files BA, McConnell ME, Strandjord SE.** Oral citrulline as arginine precursor may be beneficial in sickle cell disease: early phase two results. *J Natl Med Assoc* 93: 363–371, 2001.

Exhibit D

PubMed Health. A service of the National Library of Medicine, National Institutes of Health.

A.D.A.M. Medical Encyclopedia. Atlanta (GA): A.D.A.M.; 2011.

Hypotension

Low blood pressure; Blood pressure - low; Postprandial hypotension; Orthostatic hypotension; Neurally mediated hypotension; NMH

Last reviewed: February 20, 2011.

Low blood pressure, or hypotension, occurs when blood pressure during and after each heartbeat is much lower than usual. This means the heart, brain, and other parts of the body do not get enough blood.

See also: Blood pressure

Causes, incidence, and risk factors

Blood pressure that is borderline low for one person may be normal for another. Most normal blood pressures fall in the range of 90/60 millimeters of mercury (mm Hg) to 130/80 mm Hg. But a significant drop, even as little as 20 mm Hg, can cause problems for some people.

There are three main types of hypotension:

- Orthostatic hypotension, including postprandial orthostatic hypotension
- Neurally mediated hypotension (NMH)
- Severe hypotension brought on by a sudden loss of blood (shock), infection, or severe allergic reaction

Orthostatic hypotension is brought on by a sudden change in body position, most often when shifting from lying down to standing. This type of hypotension usually lasts only a few seconds or minutes. If this type of hypotension occurs after eating, it is called postprandial orthostatic hypotension. This form most commonly affects older adults, those with high blood pressure, and persons with Parkinson's disease.

NMH most often affects young adults and children. It occurs when a person has been standing for a long time. Children usually outgrow this type of hypotension.

Low blood pressure is commonly caused by drugs such as:

- Alcohol
- Anti-anxiety medications
- Certain antidepressants
- Diuretics
- Heart medicines, including those used to treat high blood pressure and coronary heart disease
- Medications used for surgery
- Painkillers

Other causes of low blood pressure include:

- Advanced diabetes
- Anaphylaxis (a life-threatening allergic response)
- Changes in heart rhythm (arrhythmias)
- Dehydration
- Fainting
- Heart attack

Exhibit D

- Heart failure
- Shock (from severe infection, stroke, anaphylaxis, blood loss, or heart attack)

Symptoms

Symptoms may include:

- Blurry vision
- Confusion
- Dizziness
- Fainting (syncope)
- Light-headedness
- Sleepiness
- Weakness

Signs and tests

The health care provider will examine you and try to determine what is causing the low blood pressure. Your vital signs (temperature, pulse, rate of breathing, blood pressure) will be checked frequently. You may need to stay in the hospital for a while.

The doctor will ask questions, including:

- What is your normal blood pressure?
- What medications do you take?
- Have you been eating and drinking normally?
- Have you had any recent illness, accident, or injury?
- What other symptoms do you have?
- Did you faint or become less alert?
- Do you feel dizzy or light-headed when standing or sitting after lying down?

The following tests may be done:

- Basic metabolic panel
- Blood cultures to check for infection
- Complete blood count (CBC), including blood differential
- ECG
- Urinalysis
- X-ray of the abdomen
- X-ray of the chest

Treatment

Hypotension in a healthy person that does not cause any problems usually doesn't require treatment.

If you have signs or symptoms of low blood pressure, you may need treatment. Treatment depends on the cause of your low blood pressure. Severe hypotension caused by shock is a medical emergency. You may be given blood through a needle (IV), medicines to increase blood pressure and improve heart strength, and other medicines, such as antibiotics. For more details, see the article on shock.

If you have orthostatic hypotension caused by medicines, your doctor may change the dose or switch you to a different drug. DO NOT stop taking any medicine before talking to your doctor. Other treatments for orthostatic hypotension include increasing fluids to treat dehydration or wearing elastic hose to boost blood pressure in the lower part of the body.

Those with NMH should avoid triggers, such as standing for a long period of time. Other treatments involve drinking plenty of fluids and increasing the amount of salt in your diet. (Ask your doctor about specific

Exhibit D

recommendations.) In severe cases, medicines such as fludrocortisone may be prescribed.

Expectations (prognosis)

Low blood pressure can usually be treated with success.

Complications

- Shock
- Injury from falls due to fainting

Falls are particularly dangerous for older adults. Fall-related injuries, such as a broken hip, can dramatically impact a person's quality of life.

Severe hypotension starves your body of oxygen, which can damage the heart, brain, and other organs. This type of hypotension can be life threatening if not immediately treated.

Calling your health care provider

When you have symptoms from a drop in blood pressure, you should immediately sit or lie down and raise your feet above heart level.

If low blood pressure causes a person to pass out (become unconscious), seek immediate medical treatment or call the local emergency number (such as 911). If the person is not breathing or has no pulse, begin CPR.

Call your doctor immediately if you have any of the following symptoms:

- Black or maroon stools
- Chest pain
- Dizziness, lightheadedness
- Fainting
- Fever higher than 101 degrees Fahrenheit
- Irregular heartbeat
- Shortness of breath

Also call your doctor if you have:

- Burning with urination or other urinary symptoms
- Cough with phlegm
- Inability to eat or drink
- Prolonged diarrhea or vomiting

Prevention

If you have low blood pressure, your doctor may recommend certain steps to prevent or reduce your symptoms. This may include:

- Avoiding alcohol
- Avoiding standing for a long time (if you have NMH)
- Drinking plenty of fluids
- Getting up slowly after sitting or lying down
- Using compression stockings to increase blood pressure in the legs

References

1. Calkins H, Zipes DP. Hypotension and syncope. In: Libby P, Bonow RO, Mann DL, eds. *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine*. 8th ed. Philadelphia, Pa: Saunders Elsevier;2007:chap 37.

Review Date: 2/20/2011.

Exhibit D

Reviewed by: David C. Dugdale, III, MD, Professor of Medicine, Division of General Medicine, Department of Medicine, University of Washington School of Medicine. Also reviewed by David Zieve, MD, MHA, Medical Director, A.D.A.M., Inc.

Exhibit E

PubMed Health. A service of the National Library of Medicine, National Institutes of Health.

A.D.A.M. Medical Encyclopedia. Atlanta (GA): A.D.A.M.; 2011.

Hypertension

Hypertension; HBP; Blood pressure - high

Last reviewed: June 10, 2011.

Hypertension is the term used to describe high blood pressure.

Blood pressure is a measurement of the force against the walls of your arteries as your heart pumps blood through your body.

Blood pressure readings are usually given as two numbers -- for example, 120 over 80 (written as 120/80 mmHg). One or both of these numbers can be too high.

The top number is called the systolic blood pressure, and the bottom number is called the diastolic blood pressure.

- Normal blood pressure is when your blood pressure is lower than 120/80 mmHg most of the time.
- High blood pressure (hypertension) is when your blood pressure is 140/90 mmHg or above most of the time.
- If your blood pressure numbers are 120/80 or higher, but below 140/90, it is called pre-hypertension.

If you have pre-hypertension, you are more likely to develop high blood pressure.

If you have heart or kidney problems, or if you had a stroke, your doctor may want your blood pressure to be even lower than that of people who do not have these conditions.

Causes, incidence, and risk factors

Many factors can affect blood pressure, including:

- How much water and salt you have in your body
- The condition of your kidneys, nervous system, or blood vessels
- The levels of different body hormones

You are more likely to be told your blood pressure is too high as you get older. This is because your blood vessels become stiffer as you age. When that happens, your blood pressure goes up. High blood pressure increases your chance of having a stroke, heart attack, heart failure, kidney disease, and early death.

You have a higher risk of high blood pressure if you:

- Are African American
- Are obese
- Are often stressed or anxious
- Drink too much alcohol (more than one drink per day for women and more than two drinks per day for men)
- Eat too much salt in your diet
- Have a family history of high blood pressure
- Have diabetes
- Smoke

Most of the time, no cause of high blood pressure is found. This is called essential hypertension.

Exhibit E

High blood pressure that is caused by another medical condition or medication is called secondary hypertension. Secondary hypertension may be due to:

- Chronic kidney disease
- Disorders of the adrenal gland (pheochromocytoma or Cushing syndrome)
- Pregnancy (see: preeclampsia)
- Medications such as birth control pills, diet pills, some cold medications, and migraine medications
- Narrowed artery that supplies blood to the kidney (renal artery stenosis)
- Hyperparathyroidism

Symptoms

Most of the time, there are no symptoms. For most patients, high blood pressure is found when they visit their health care provider or have it checked elsewhere.

Because there are no symptoms, people can develop heart disease and kidney problems without knowing they have high blood pressure.

If you have a severe headache, nausea or vomiting, bad headache, confusion, changes in your vision, or nosebleeds you may have a severe and dangerous form of high blood pressure called malignant hypertension.

Signs and tests

Your health care provider will check your blood pressure several times before diagnosing you with high blood pressure. It is normal for your blood pressure to be different depending on the time of day.

Blood pressure readings taken at home may be a better measure of your current blood pressure than those taken at your doctor's office. Make sure you get a good quality, well-fitting home device. It should have the proper sized cuff and a digital readout.

Practice with your health care provider or nurse to make sure you are taking your blood pressure correctly. See also: Blood pressure monitors for home

Your doctor will perform a physical exam to look for signs of heart disease, damage to the eyes, and other changes in your body.

Tests may be done to look for:

- High cholesterol levels
- Heart disease, such as an echocardiogram or electrocardiogram
- Kidney disease, such as a basic metabolic panel and urinalysis or ultrasound of the kidneys

Treatment

The goal of treatment is to reduce blood pressure so that you have a lower risk of complications. You and your health care provider should set a blood pressure goal for you.

If you have pre-hypertension, your health care provider will recommend lifestyle changes to bring your blood pressure down to a normal range. Medicines are rarely used for pre-hypertension.

You can do many things to help control your blood pressure, including:

- Eat a heart-healthy diet, including potassium and fiber, and drink plenty of water. See: High blood pressure and diet
- Exercise regularly – at least 30 minutes of aerobic exercise a day.
- If you smoke, quit – find a program that will help you stop.
- Limit how much alcohol you drink – one drink a day for women, two a day for men.
- Limit the amount of sodium (salt) you eat – aim for less than 1,500 mg per day.
- Reduce stress – try to avoid things that cause you stress. You can also try meditation or yoga.
- Stay at a healthy body weight – find a weight-loss program to help you, if you need it.

Your health care provider can help you find programs for losing weight, stopping smoking, and exercising. You

Exhibit E

can also get a referral from your doctor to a dietitian, who can help you plan a diet that is healthy for you.

There are many different medicines that can be used to treat high blood pressure. See: High blood pressure medicines

Often, a single blood pressure drug may not be enough to control your blood pressure, and you may need to take two or more drugs. It is very important that you take the medications prescribed to you. If you have side effects, your health care provider can substitute a different medication.

Expectations (prognosis)

Most of the time, high blood pressure can be controlled with medicine and lifestyle changes.

Complications

When blood pressure is not well controlled, you are at risk for:

- Bleeding from the aorta, the large blood vessel that supplies blood to the abdomen, pelvis, and legs
- Chronic kidney disease
- Heart attack and heart failure
- Poor blood supply to the legs
- Stroke
- Problems with your vision

Calling your health care provider

If you have high blood pressure, you will have regular appointments with your doctor.

Even if you have not been diagnosed with high blood pressure, it is important to have your blood pressure checked during your yearly check-up, especially if someone in your family has or had high blood pressure.

Call your health care provider right away if home monitoring shows that your blood pressure is still high.

Prevention

Adults over 18 should have their blood pressure checked regularly.

Lifestyle changes may help control your blood pressure.

Follow your health care provider's recommendations to modify, treat, or control possible causes of high blood pressure.

References

1. Goldstein LB, Bushnell CD, Adams RJ, Appel LJ, Braun LT, Chaturvedi S, et al. Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2011 Feb;42:517-84.
2. Kaplan NM. Systemic hypertension: Treatment. In: Bonow RO, Mann DL, Zipes DP, Libby P, eds. *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine*. 9th ed. Philadelphia, Pa: Saunders Elsevier; 2011:chap 46.
3. Victor, RG. Systemic hypertension: Mechanisms and diagnosis. In: Bonow RO, Mann DL, Zipes DP, Libby P, eds. *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine*. 9th ed. Philadelphia, Pa: Saunders Elsevier; 2011:chap 45.

Review Date: 6/10/2011.

Reviewed by: David C. Dugdale, III, MD, Professor of Medicine, Division of General Medicine, Department of Medicine, University of Washington School of Medicine. Also reviewed by David Zieve, MD, MHA, Medical Director, A.D.A.M., Inc.

Current strategies for the management of neonatal urea cycle disorders

Marshall Summar, MD

The treatment of newborns with urea cycle disorders has evolved over the years into a complex multidisciplinary effort. The complexity derives from the number of issues that must be addressed simultaneously. At the Urea Cycle Disorders Consensus Meeting held in Washington, D.C., a panel of physicians and other professionals with extensive experience in this field was assembled to bring some systematization to this task. This manuscript is a condensation of the collective opinion and experience of that group. The outcome of untreated or poorly treated patients with urea cycle disorders is universally bad. Although a favorable outcome is not always feasible, even with the best therapy, the methods outlined here should help treat such a patient by drawing on the experience of others who have treated patients with urea cycle disorders. This article does not purport to be the final word in treating children with these disorders. However, by establishing some common ground, new methods can be tried and compared with existing ones. In a future that holds the prospect of gene therapy "cures" for these diseases, striving for the best possible outcome in the critical newborn period is a worthy goal. (J Pediatr 2001;138:S30-S39)

Neonatal hyperammonemia is a medical emergency requiring advanced planning, sophisticated facilities, and multidisciplinary teamwork. Urea cycle disorders are the primary cause of hyperammonemia during the vulnerable newborn period. Genetic defects in any of the first 4 enzymes of the pathway (carbamyl phosphate synthetase I, ornithine transcarbamylase, argininosuccinic acid synthetase, argininosuccinic acid lyase), or a cofactor producer (N-acetyl glutamate synthase) result in accumulations of precursor metabolites including ammonia (Fig 1). Because there is no effective secondary clearance system for ammonia, disruption of this pathway has a rapid clinical course. The catabolism normally present in the newborn period together with the immaturity of the liver combine to accentuate defects in these enzymes. This rapid accumulation of ammonia and other precursor metabolites results in acute cerebral edema with severe neurologic compromise.[1-3] Thus fast and effective treatment is key to improving the patient's outcome.

A clear, concise protocol is required to treat neonates with severe hyperammonemia caused by UCDs. In reviewing the experience of a number of clinicians who have cared for these patients, several stages of treatment become apparent. These include (1) recognition and supportive treatment, (2) bulk ammonia removal and pharmacologic scavenging, (3) stabilization and catabolic reversal, and (4) transition to home management. These steps are undertaken to accomplish specific therapeutic goals and include rapidly clearing ammonia from the neonate's bloodstream, blocking the production of additional ammonia, removing excess nitrogen, and protecting the neurologic integrity of the baby. All of these goals should be pursued with thoughtful expediency in the context of the patient's clinical situation.

ASL	Argininosuccinic acid lyase
ASS	Argininosuccinic acid synthetase
CPS	Carbamyl phosphate synthetase I
ECMO	Extracorporeal membrane oxygenation
ECMO/HD	Extracorporeal membrane oxygenation driving a hemodialysis machine
NAGS	N-acetyl glutamate synthase
NG	Nasogastric
NJ	Nasojejunal
OTC	Ornithine transcarbamylase
UCD	Urea cycle disorder

During each stage of management there are a number of critical elements to consider, including what is being done, what are the results, and what remains to be done. This article is an attempt to provide guidance on the

From the Division of Medical Genetics, Department of Pediatrics and Molecular Physiology & Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee.

Reprint Requests: Marshall Summar, MD, Division of Medical Genetics, Department of Pediatrics, Vanderbilt University Medical Center, DD 2205 MCN, Nashville, TN 37232-2578.

0022-3476/2001/$35.00 + 0 9/0/111834

doi:10.1067/mpd.2001.111834

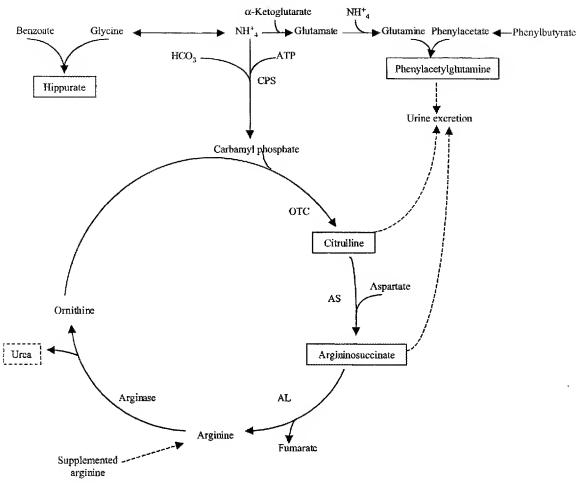


Fig 1. Urea cycle and intermediate components.

specifics of treating a patient with neonatal hyperammonemia.

RECOGNITION AND SUPPORTIVE TREATMENT

Once neonatal hyperammonemia is recognized, the necessary organization and supportive care are initiated to reverse it as soon as possible.

Clinical Presentation

In the immediate newborn period, infants with UCDs will typically look normal. The problems that may have been observed while the child was still in hospital are often not seen until the child is at home because of the current practice of discharging the mother and newborn baby early. This places much of the burden of recognition on the family and the pediatrician or primary care physician. The typical initial symptoms of a child with hyperammonemia, failure to feed, and somnolence may not be recognized by new parents. As a result, advice and care is sought later when the child's illness has progressed to become more severe.

The progression of symptoms moves from somnolence, through lethargy, and on to coma. There is a loss of thermoregulation with a low core temperature and feeding disruption that correlates with the somnolence.

Abnormal posturing and encephalopathy are often related to the degree of central nervous system swelling and pressure on the brain stem.[4,5] Seizures are seen in approximately 50% of severely hyperammonemic neonates. Hyperventilation caused by cerebral edema causes a respiratory alkalosis that is also a common symptom in the early stages of the hyperammonemic attack. This progresses to hypoventilation and respiratory arrest as pressure increases on the brain stem.[6,7]

The algorithm in Fig 2 may assist with the evaluation of a hyperammonemic newborn, but outside factors can influence the differential diagnosis. Factors such as the overall health of the liver, the duration of hyperammonemia, and pharmacologic agents already given to the patient should be factored into the interpretation of the clinical observations.

Diagnostic Considerations

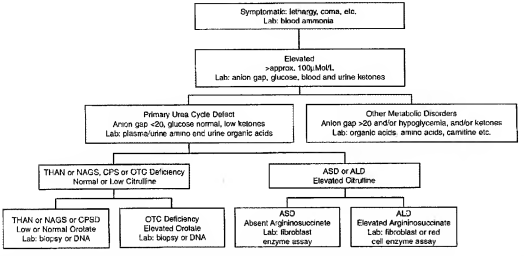


Fig 2. Algorithm for encephalopathic newborns.

Table I. Diagnostic laboratory tests

Ammonia
pH and CO_2
Plasma quantitative amino acids
Anion gap
Glucose
Urine organic acids and orotic acid
Specific enzymatic or DNA analysis

Table II. Laboratory measurements in acutely ill infants without UCD

Parameter	Average	SD
Ammonia	47 µmol/L	(13.5)
Glutamine	581	(182)
Glycine	302	(100)
Alanine	319	(252)
Citrulline	10.3	(7)
Arginine	38.3	(18.4)

Averaged results from 25 babies admitted to the neonatal intensive care unit.
All babies were 35 weeks' gestation with respiratory problems (birth asphyxia, respiratory distress, or meconium aspiration syndrome).

Laboratory data useful in the diagnosis of UCDs include plasma ammonia levels, pH, CO_2, the anion gap, plasma amino acids, and urine organic acid analyses. Table I lists the recommended diagnostic tests, and Fig 2 highlights their use. The clinician should remember that treatment should begin before a final diagnosis is made, and that later stages of treatment should be tailored to the specific disorder (Table I).

An elevated plasma ammonia level of 150 µmol/L or higher, associated with a normal anion gap and a normal blood glucose level, is a strong indication for the presence of a UCD. Quantitative amino acid analysis can be used to evaluate these patients and arrive at a tentative diagnosis. The amino acids in sick newborns are often quite different from those in children and adults, and Table II lists some of our averaged values obtained in sick, term newborns without UCDs. The required diagnostic laboratory tests and their interpretation are discussed in more detail elsewhere in this supplement.

In summary, infants with a UCD often have an initial normal appearance that progresses to lethargy and coma with the associated features of anorexia, hyperventilation, hypothermia, hypoventilation, seizures, neurologic posturing, and other features of cerebral edema.

Early Supportive Care

These are the initial treatment steps that should be implemented as soon as the patient is suspected of having a urea cycle defect. They can be performed while the patient is being prepared for transport to a metabolic center or being prepared for dialysis or pharmacologic management. Before care is initiated, some thought should be given to the severity of the patient's condition and to the probable long-term outcome. Patients who have been in a hyperammonemic coma for several days have an extremely poor neurologic outcome. Although the patient may be successfully "detoxified" and stabilized, the damage to the central nervous system is likely to be devastating and permanent. Thus the option of withdrawal of support should be dis-

cussed with parents of patients who have already sustained overwhelming damage to the brain.

Intravenous access should be established as soon as possible. If practical, the patient with a UCD should have a deep line placed such as an umbilical catheter or a multilumen central line. The need to resume feedings rapidly should influence the selection of line type. Stable vascular access will assist with the administration of fluids, medications, and the frequent blood sampling. Many patients with UCDs are dehydrated at presentation as a result of anorexia and poor oral intake. Restoration of normal hydration will serve to protect renal function (critical for effective treatment) and ensure adequate tissue perfusion (to blunt the further catabolic production of nitrogen). Overhydration should be avoided because most patients with UCDs have some degree of cerebral swelling. Intravenous administration of fluids with 10% dextrose with one quarter normal saline solution is preferable to physiological saline solution, because patients treated with ammonia-scavenging drugs will receive large amounts of sodium and chloride ions as part of their medication regimen. Other support (pressors, buffering agents), depending on the cardiovascular and acid-balance status of the infant, is also important. Protection of kidney function is an important aspect of the early treatment of these patients. Many have depleted intravascular volumes and go into shock and have acute renal failure. Once the dialysis phase is complete, the drugs used to scavenge excess ammonia from the bloodstream require normal renal function. The use of boluses of fluid and pressor agents should be balanced against the degree of cerebral edema present at the time. Oncotic agents such as albumin will contribute to the overall nitrogen load but in selected cases, and on a limited basis, can be used.

Caloric supplementation should be maximized to try and reverse catabolism and nitrogen turnover. In addition to glucose, Intralipid administration can provide additional calories but should not be allowed to delay progress toward more aggressive treatment. Oral feedings should be discontinued in patients with severe encephalopathy but restarted as soon as practical. Placement of a nasogastric tube should be done during this early phase. Feeding of all protein should be halted temporarily and calories provided as carbohydrate and fat. For patients who are able to tolerate oral feedings, a protein-free formula such as Mead Johnson 80056 or Ross Formula ProPhree could initially be used. Elemental formulas are not appropriate because they contain considerable amounts of nitrogen. This complete restriction of protein should be maintained only for a short period (24 to 48 hours), because depletion of essential amino acids will result in further protein catabolism and nitrogen release. The author has found that maximizing caloric intake has a significant impact on patient stabilization after bulk ammonia removal.

Even an infant who is awake and responsive can progress to coma and cardiovascular or respiratory collapse during transport or preparation for dialysis. Therefore it is preferable to perform intubation on infants with borderline clinical condition before transport or before they have respiratory compromise for 2 reasons: (1) if a patient is breathing rapidly (respiratory alkalosis driven by cerebral edema), excess calories are burned, contributing to catabolism and further nitrogen accumulation, and (2) intubation is a difficult procedure to carry out while an infant is being transported and can lead to hypoxia.

It is usual for a lethargic infant to have undergone a septic workup with the initiation of antibiotic treatment. With the heavy instrumentation and stress patients with UCD undergo, it is probably prudent to continue existing antibiotic coverage or consider initiating it as prophylaxis. A bacterial infection in a newborn baby with hyperammonemia could well prove fatal.

There are several other important measures to be taken when caring for these infants. Hyperventilation is recommended and steroids are to be avoided, because they will increase the amount of protein turnover and hence increase the nitrogen load. Mannitol has not been demonstrated to be effective in managing cerebral edema caused by hyperammonemia.

The importance of early treatment cannot be overstressed. Ultimately the neurologic dysfunction of the patient is related to the duration of cerebral edema.[7-10] Most children will have cognitive impairment, but early treatment to remove ammonia and other metabolites from the bloodstream will lessen the severity of this impairment.

Organization and Mobilization

Newborns with UCDs should be treated by a team of experienced personnel and in facilities with special resources (Table III). The community physicians should be aware of these facilities and how to reach them. A metabolic specialist should coordinate the activities of the various team members and maintain continuity of treatment. As with any team approach, the roles of the members and the steps and goals of treatment should be clear before a patient with a UCD presents for treatment. In addition to alerting team members of the impending arrival of a patient with a UCD, the managing physician should also alert the laboratory regarding STAT tests. The pharmacy should also be alerted to ensure that the specific medications are available and can be prepared at short notice. Human subject permits should already be on hand, because treatment with intravenous sodium phenylacetate and sodium benzoate is still considered experimental and is under an FDA investigational new drug permit (contact Ucyclyd Pharmaceuticals for details). We have found that having

Table III. Treatment team members and roles and responsibilities

Team member	Roles and responsibilities
Metabolic specialist	Coordinate treatment and management.
Pharmacy	Formulate ammonia scavenging and dialysis agents. Check dosing orders.
Nephrologist or dialysis team	Dialysis
Intensive care team	Assist with physiological support, pain management, and ventilator management.
Surgical team	Catheter placement for hemo- and peritoneal dialysis. Obtain biopsy sample for diagnostic testing.
Laboratory staff	Ammonia, amino acids, and organic acids
Nutritionist	Establish dietary prescription with metabolic foods and supplements. Assist with parenteral calorie management and transition to enteral feeding.

the medications from the pharmacy ahead of time and having blood hand-delivered to the chemistry laboratory saves considerable critical time. Delays in treatment or response to changing status may affect the eventual outcome (Table III).

Bulk Ammonia Removal and Pharmacologic Ammonia Scavenging

The best way to remove ammonia rapidly is by dialysis.[7,11-13] Keeping ammonia from reaccumulating is achieved through the use of nitrogen scavenger drugs, discussed in detail elsewhere in this supplement. Loading with scavenger drugs should be done as soon as possible if urine output is adequate. Exchange transfusion is ineffective in removing ammonia, and dialysis is the treatment of choice for rapid removal of this toxin.[7,11-13]

Preparation for Dialysis

Dialysis is the primary means by which ammonia is removed from the patient's body during the early management period. Ideally, the surgical and dialysis teams should be waiting for the patient to arrive and initiate treatment immediately. If dialysis is not immediately available, it is appropriate to use a loading dose of drugs to induce the removal of ammonia. However, in the patient with severe hyperammonemia, pharmacologic agents are not sufficient to remove ammonia quickly.

The method of dialysis chosen depends on the available expertise and equipment. The fastest removal system uses an extracorporeal membrane oxygenation pump system to drive a hemodialysis machine.[12,14,15] ECMO has become more widely available because of its use in infant lung disease and cardiac surgery. Other methods include hemofiltration (both arteriovenous and venovenous), standard dialysis, peritoneal dialysis, and continuous-drainage peritoneal dialysis. Each method has its own advantages and drawbacks.[12,14-18] Because ammonia crosses the dialysis membrane rapidly, the faster the flow rate, the higher the clearance. In critically ill newborns it is difficult to perform standard dialysis for more than a few hours and maintain homeostasis. Peritoneal dialysis clears ammonia at a low rate of 3 to 5 mL/min, and if it is the sole means of ammonia removal, it may take several days to reduce a significant ammonia burden.[12] Peritoneal dialysis also complicates attempts at early refeeding and increases the risk of infection. However, peritoneal dialysis may be the most widely available form of toxin removal and does not require an entire dialysis team. A variation of peritoneal dialysis with continuous inflow and outflow is effective but requires extremely close monitoring. Hemofiltration produces clearance rates of 10 to 30 mL/min, and clearance rates with ECMO/HD are on the order of 170 to 200 mL/min.[12] With the advent of percutaneous catheter placement for ECMO and the increased pump rates available, the advantages of this method may outweigh the risk of blood vessel damage. Another advantage to the use of an ECMO pump is that a hemofilter can be placed in the circuit to continue removal of ammonia between dialysis runs. We have demonstrated reductions of blood ammonia levels by >1000 μmol/L in a period of 1 to 2 hours with ECMO/HD.[12] Osmotic shifts have not been observed with this rapid dialysis, and recovery of neurologic activity is faster. For patients with less severe hyperammonemia, a hemofiltration pump may suffice for bulk ammonia removal. A review of the literature suggests that approximately 50% of the neonates requiring dialysis for any reason undergo dialysis with a pressure-supported system. The extensive amount of instrumentation arising from the use of any of these methods increases the risk of infection, and prophylactic antibiotic coverage should be considered for all patients.

Dialysis seems to become less effective when the plasma ammonia level falls below 200 μmol/L and can be discontinued. Once dialysis is stopped, and while the patient is still in the acute phase, there may be a rebound of several hundred μmol in the ammonia level. This reflects both the continued catabolic state of the patient with the consequent production of waste nitrogen and the time required for the nitrogen scav-

enging medications to take effect. The use of hemofiltration can blunt or prevent this rebound. It is recommended that the catheters be removed only after the plasma ammonia level has been stable for at least a day. However, this should be balanced by the risk of maintaining the patient in an anticoagulated state. Some patients may require more than one session of dialysis to bring the ammonia level under control. In summary, it is recommended that patients with severe hyperammonemia be treated initially with pump-driven dialysis (ECMO/HD) followed by continuous hemofiltration with the same pump system. In patients who are hemodynamically unstable, this procedure offers pressure support and better control over dialysis flow rates. A further advantage is that enteral feeding through an NG or nasojejunal tube can be started.

Pharmacologic Management

RATIONALE. The second line of the treatment for acute hyperammonemia involves the use of compounds that remove nitrogen by alternative pathways. Phenylacetate combines with glutamine to produce phenylacetylglutamine, a compound that is excreted in the urine.[8,19] The scavenged glutamine is replaced by synthesis in muscle and liver, thus reducing the nitrogen load. In addition, glutamine has been implicated in the neurotoxicity of UCDs; therefore its elimination may have a more direct beneficial effect.[20-23] Benzoate combines with glycine to produce hippurate, which is also rapidly cleared by the kidneys.[24,25] The glycine is replaced by synthesis, thus removing more waste nitrogen from the pool. While the precursor nitrogen is removed from circulation, attention must also be paid to replacing the deficient product(s) of the urea cycle (Fig 1). CPS, NAGS, and OTC defects prevent the formation of citrulline from ornithine and carbamyl phosphate. This in turn decreases the synthesis of arginine, resulting in it becoming an essential amino acid. A block in ASS prevents the condensation of aspartate with citrulline, which accounts for 50% of the nitrogen incorporated into the pathway. ASL deficiency blocks conversion of argininosuccinate to arginine. Therefore arginine is also an essential amino acid in ASS and ASL deficiencies.[26,27] Even in ASS and ASL deficiency, where there is a partially intact urea cycle, the body rapidly depletes its pool of urea cycle intermediates into which it normally incorporates nitrogen. Therefore arginine serves as a therapeutic agent in UCD. In CPS, NAGS, OTC, ASS, and ASL deficiency, arginine is used to restore its blood levels and prevent the breakdown of endogenous protein.[26,27] In ASS and ASL deficiency it is used in larger amounts to "prime" the cycle to produce citrulline or argininosuccinate.[24,28] This has the advantage of incorporating a substantial amount of nitrogen in compounds having a lower toxicity and higher renal excretion. In ASS (citrullinemia), 1 mol of nitrogen can be removed for every mole of arginine metabolized through the cycle, and this doubles in ASL to 2 mol. L-citrulline may serve as a better supplement for patients with OTC and CPS than arginine, because it is converted to arginine while promoting the incorporation of one waste nitrogen; however, an intravenous formulation of citrulline is not currently available. A note of caution concerning arginine. Arginine is a precursor of nitric oxide, a potent vasodilator. Anecdotal evidence and the author's own experience suggest that in CPS and OTC deficiencies, large amounts of excess arginine may accumulate, resulting in overproduction of nitric oxide and leading to extreme vasodilation and hypotension.[12] Reduction in arginine administration corresponded with restored venous tone in 2 patients cared for in our institution.

Administration Protocol

These dose recommendations are based on the Food and Drug Administration packaging for intravenous sodium benzoate/sodium phenylacetate and the protocols developed by Dr. Saul Brusilow.[6,7,11] They are summarized in Table IV.

Loading Dose

The current protocol for the acute management of hyperammonemia includes arginine hydrochloride (600 mg/kg in a 10% solution), and a combination of sodium benzoate and sodium phenylacetate (250 mg/kg of each drug), all infused in 25 to 35 mL/kg of 10% dextrose in water over a 90-minute period. The blood pH should be monitored and buffer added to counteract the acidity of the arginine hydrochloride. The dose of arginine has been increased from previous protocols, because a rapid infusion of arginine is believed to have a significant impact on patients with ASS and ASL deficiency and be relatively safe for patients with OTC, CPS, and NAGS deficiency. Caution should be exercised if additional doses of sodium benzoate and sodium phenylacetate are given within 24 hours of the original dose, but experience suggests that 500 mg/kg each of sodium benzoate and sodium phenylacetate during the first 24 hours is an acceptable regimen (1 loading dose + maintenance infusion). At 750 mg/kg/24 h, there is some toxicity (vomiting, lethargy), and at >750 mg/kg/24 h, the toxicity is essentially invariable and can be life-threatening. Reloading (repeating the initial regimen) should be contemplated only in neonates with severe disorders or those who are undergoing dialysis and, based on the results of pharmacokinetic studies, should be spaced at least 6 hours apart (refer to Dr. Batshaw's article elsewhere in this supplement for more extensive information on the pharmacologic management of UCDs).

Maintenance Infusion

Once the loading dose is given, the patient should be switched to the maintenance infusion. The doses differ only in

Table IV. Recommended doses of pharmacologic agents to be used during dialysis periods

Sodium benzoate/phenylacetate dosage and administration
Summary table

Patient population	Components of infusion solution			Dosage provided		
	SB/SA Injection, 10%	Arginine HCl Injection, 10%	Dextrose injection, 10%	Sodium phenylacetate	Sodium benzoate	Arginine Hcl
Neonates/Infants/Young children:						
Prospective treatment pending definitive diagnosis of urea cycle enzyme deficiency						
Loading dose	2.5 mL/kg	6.0 mL/kg	~ 25 mL/kg	250 mg/kg	250 mg/kg	600 mg/kg
Maintenance dose	2.5 mL/kg	6.0 mL/kg	~ 25 mL/kg	250 mg/kg	250 mg/kg	600 mg/kg
CPS or OTC Deficiency						
Loading dose	2.5 mL/kg	2.0 mL/kg	~ 25 mL/kg	250 mg/kg	250 mg/kg	200 mg/kg
Maintenance dose	2.5 mL/kg	2.0 mL/kg	~ 25 mL/kg	250 mg/kg	250 mg/kg	200 mg/kg
ASS or ASL deficiency						
Loading dose	2.5 mL/kg	6.0 mL/kg	~ 25 mL/kg	250 mg/kg	250 mg/kg	600 mg/kg
Maintenance dose	2.5 mL/kg	6.0 mL/kg	~ 25 mL/kg	250 mg/kg	250 mg/kg	600 mg/kg
Older children and adults:						
CPS or OTC deficiency						
Loading dose	55 mL/m^2	2.0 mL/kg	~ 25 mL/kg	5.5 g/m^2	5.5 g/m^2	200 mg/kg
Maintenance dose	55 mL/m^2	2.0 mL/kg	~ 25 mL/kg	5.5 g/m^2	5.5 g/m^2	200 mg/kg
ASS or ASL deficiency						
Loading dose	55 mL/m^2	6.0 mL/kg	~ 25 mL/kg	5.5 g/m^2	5.5 g/m^2	600 mg/kg
Maintenance dose	55 mL/m^2	6.0 mL/kg	~ 25 mL/kg	5.5 g/m^2	5.5 g/m^2	600 mg/kg*

SB, Sodium benzoate; *SA*, sodium phenylacetate.

the amount of arginine hydrochloride given and are dependent on the diagnosis. The 24-hour dose of the combination of sodium benzoate/sodium phenylacetate is 250 mg/kg/24 h of each drug. For patients with CPS, OTC, or NAGS deficiency, the dose of arginine hydrochloride is 200 mg/kg/24 h. For ASS and ASL deficiency the dose of arginine is 600 mg/kg/24 h. For patients awaiting diagnosis, the 200 mg/kg/24 h dose of arginine should be used in conjunction with sodium benzoate and sodium phenylacetate. Attention should be paid to the potassium level of the patient, and the maintenance fluids should have potassium added, because sodium phenylacetate may cause potassium depletion. The maintenance infusion is continued until a conversion to oral medication is made. Cerebral blood flow or electroencephalography analysis may be required to determine whether treatment should be discontinued.

Laboratory Monitoring

Our center measures ammonia plasma levels every hour during high-flow rate dialysis. These samples should be handled expeditiously to get the fastest possible turnaround. Once the ammonia plasma level has stabilized to <200 to 300 μmol/L, the monitoring frequency can be reduced to once every few hours. When the patient is receiving a stable drug regimen and no longer requires dialysis, the frequency can be further reduced to every 12 hours and to once-daily before discharge. During the acute phase, electrolytes and acid-base balance should be carefully monitored (every 4 hours). Glucose should be monitored hourly in patients receiving a glucose/insulin infusion to avoid wide swings in glucose levels. Amino acids should be monitored on a daily basis to assess nutritional status and the effectiveness of glutamine removal and citrulline/arginine replacement. The amount of blood removed should be monitored and transfusion used to avoid iatrogenic anemia.

Other Acute Treatment Issues

Opinion is divided among experts on the use of glucose and insulin in these patients. Glucose and insulin can serve as suppressors of catabolism, but their use requires care. The consensus opinion at this meeting was to administer 6 to 8 mg/kg/min of glucose (administered as 10% dextrose in water) and to use insulin sparingly to maintain the serum glucose level <170 mg/dL. The presence of glycosuria is an indication for continued administration of intravenous regular insulin at a rate that keeps glucose levels between 120 and 170 mg/dL. Wide swings in glucose levels can change the osmolality of the brain and therefore should be avoided.

The use of normal saline solution should also be avoided, because the pharmacologic agents used in ammonia removal contain large amounts of sodium and chloride ions.

The use of carnitine in neonates being treated with sodium benzoate is not believed to be beneficial. There have been no documented cases of carnitine deficiency, even though carnitine levels are low in these neonates, and carnitine is known to conjugate with sodium benzoate.

Valproic acid should be avoided in any patient who has seizures. It is known to decrease urea cycle function and will aggravate the hyperammonemia.[29-31]

Enteral citrulline is used in some centers for neonates with UCD and CPS or OTC deficiencies, the rationale being that pulling aspartate into the pathway may increase nitrogen clearance. The dose of citrulline used is 150 to 200 mg/kg/24 h. A clear diagnosis should be made before citrulline is used to avoid providing citrulline to patients with ASS and ASL who already have excessive amounts of this amino acid.

Stabilization and Catabolic Reversal

This phase overlaps with the second phase to some extent. One of the keys to successful treatment of a patient with a UCD is to reverse the catabolic process. This decreases nitrogen turnover and reduces the amount of ammonia that must be detoxified. During this stage there may be fluctuations in the ammonia plasma levels, and often the bulk ammonia removal protocol will have to be repeated. Some form of low-level dialysis (hemofiltration) may be continued during the early parts of this phase while the patient's nitrogen production exceeds removal.

Caloric Management

The author recommends that the patient be started on some form of enteral feed as soon as is practical. This may occur while the patient is still undergoing cannulation. The placement of an NG or NJ tube may facilitate this. Parenteral nutrition (with reduced amino acid content) should be maximized during the acute phase to transition the patient to enteral feeds. The highest infusion rate of glucose (dextrose) that does not cause hyperglycemia should be used. Intralipid should also be used to maximize parenteral calories. The use of Intralipid may provide a rationale for the use of carnitine to improve cellular transport of fatty acids. Essential amino acids should not be withheld for >24 hours. Most patients with a UCD are nutritionally compromised on admission to the hospital. Although the addition of excess nitrogen in the form of amino acids and protein should be avoided, essential amino acids will be released from the patient's own protein stores if they are not provided, thus increasing the amount of waste nitrogen requiring disposal. Nutritional intake should be managed with the active participation of a nutritionist experienced in metabolic disorders. Targets of 1.0 to 1.5 g of protein/kg body weight (50% as essential amino acids) are a good start during the early phases of treatment. Dr. Leonard's article in this supplement discusses the nutritional treatment of patients with these disorders in more detail. A number of commercial products are available to treat these patients including protein-free formulas (Mead Johnson 80056, Ross ProPhree) and essential amino acid formulations such as SHS UCD-1 and Ross Cyclinex-1.

At this stage the patient's neurologic status should be assessed in response to treatment and the degree of impairment that may have occurred from the disease.

Once the patient is stabilized, feedings have been established, and the ammonia level is not fluctuating, the patient can be switched to the oral formulations of the nitrogen-scavenging medications. Dr. Batshaw's article in this supplement discusses long-term pharmacologic management in greater detail. Current dosing recommendations for newborn patients with CPS or OTC deficiency are sodium phenylbutyrate (450 to 600 mg/kg/d) and citrulline (170 mg/kg/d). Patients with ASS deficiency receive sodium phenylbutyrate (450 to 600 mg/kg/d) and arginine (400 to 700 mg/kg/d). Patients with ASL deficiency receive L-arginine (400 to 700 mg/kg/d), and if their hyperammonemia is not controlled by diet and arginine alone, some patients also receive sodium phenylbutyrate. Of note, some patients with ASL deficiency may have significant hepatomegaly and chronic elevation of transaminases leading to varying degrees of fibrosis. The reason for this is not clearly understood, although in patients treated by the author, it does not appear to significantly affect hepatic function. Some clinicians also use sodium benzoate at a dose of 250 to 500 mg/kg/d if sodium phenylbutyrate is not sufficient to scavenge the excess nitrogen. Sodium benzoate is a caustic substance and will cause a rash if it comes into contact with skin, and a burn reaction if extravasated from an intravenous site.

TRANSITION TO HOME MANAGEMENT

During this phase preparations are made to facilitate the continuing treatment of the patient in the home environment.

Careful consideration should be given to the route of feeding. Patients with severe neurologic impairment usually require the placement of a gastrostomy tube. This is probably safer than repeatedly passing an NG tube and is less stressful for the family. Inadequate protein supplementation may prevent healing of the insertion site with frequent skin breakdown and should be monitored. Placement of the tube before initial discharge may pre-

vent a later admission for nutritional failure. A metabolic nutritionist should build a rapport with the family, because the most stressful issues at home usually center on feeding. A supply of the special formulas must be identified and insurance coverage issues managed. Letters of justification will typically be required for the formulas and medications. Arginine and citrulline are medical supplements rather than Food and Drug Administration drugs, and explanatory documentation will usually be necessary.

The introduction of a patient with a UCD into most families is a very stressful event, and support for the family should be arranged. Most states have programs for home visits by medical professionals, and families with a child with a UCD often benefit from them. The author often recommends prospective family counseling to deal with the changes in dynamics before they become critical. Time also must be spent with close relatives to explain an illness with which few of them will be familiar. Patients with a UCD are perceived as extremely fragile by parents and relatives, and the family should be reassured that physically these children are not more delicate than others. However, exposure to individuals with contagious illness (eg, upper respiratory infections, gastroenteritis) should be minimized.

The patient should follow a normal immunization schedule, and antipyretics can be used prophylactically. A multivitamin should also be used, although the medical foods are well compounded for vitamins and trace elements. Often the formulas are mixed with bottled or sterile water that does not contain fluoride, and some provision for fluoride treatment may be needed. The author has observed the development of extensive dental caries in a number of patients who did not receive fluoride supplementation. The pediatrician or primary care provider plays a crucial role in caring for a patient with a UCD and should be involved well before the patient's discharge. It is useful to prepare a treatment sheet for medical emergencies and to keep it updated at clinic visits. This can save crucial time and prevent confusion.

If liver transplantation is being considered for the patient, the appropriate laboratories and contacts should be made before the patient's discharge.

CONCLUDING REMARKS

The treatment of patients with a urea cycle disorder presents one of the most challenging and rewarding tasks in metabolic disease. The best outcome for the patient can be obtained only by careful planning, coordination, and thought. The stress to the patient can be minimized by planning the transitions between early management, ammonia removal, and stabilization. The combination of physical and pharmacologic removal must be monitored very closely, because each patient will react differently. Advanced preparation is very important, because it is virtually impossible to assemble all of the resources to treat a patient with UCD without preplanning. This preparation includes identification of a laboratory that can rapidly process and report plasma amino acids to confirm the diagnosis and plasma ammonia values to assist with real-time management. Advance planning also means that the roles of potential members of the treatment team are known well in advance. As with all detailed treatment schemes, the clinician must maintain a high degree of adaptability to allow a fast response to the specific patient circumstances. This can be facilitated by contacting others with experience in treating patients with these disorders. A consensus article like this one is only possible with the use of the accumulated knowledge and experience of a large number of individuals who have pioneered this field. As Sir Isaac Newton said, "If I have seen farther than others, it is because I was standing on the shoulders of giants."

REFERENCES

1. Bachmann C. Treatment of congenital hyperammonemias. Enzyme 1984;32: 56-64.
2. Batshaw ML. Hyperammonemia. Curr Probl Pediatr 1984;14:1-69.
3. Farriaux JP, Ponte C, Pollitt RJ, Lequien P, Formstecher P, Dhondt JL. Carbamyl-phosphate-synthetase deficiency with neonatal onset of symptoms. Acta Paediatr Scand 1977;66: 529-34.
4. Butterworth RF, Giguere JF, Michaud J, Lavoie J, Layrargues GP. Ammonia: key factor in the pathogenesis of hepatic encephalopathy. Neurochem Pathol 1987;6:1-12.
5. Butterworth RF. Effects of hyperammonaemia on brain function. J Inherit Metab Dis 1998;21(Suppl 1):6-20.
6. Brusilow SW. Urea cycle disorders: clinical paradigm of hyperammonemic encephalopathy. Prog Liver Dis 1995; 13:293-309.
7. Brusilow SW, Maestri NE. Urea cycle disorders: diagnosis, pathophysiology, and therapy. Adv Pediatr 1996;43: 127-70.
8. Batshaw ML, Brusilow SW. Treatment of hyperammonemic coma caused by inborn errors of urea synthesis. J Pediatr 1980;97:893-900.
9. Batshaw ML. Inborn errors of urea synthesis. Ann Neurol 1994;35:133-41.
10. Brusilow SW. Disorders of the urea cycle. Hosp Pract (Off Ed) 1985;20: 65-72.
11. Batshaw ML, Monahan PS. Treatment of urea cycle disorders. Enzyme 1987; 38:242-50.
12. Summar M, Pietsch J, Deshpande J, Schulman G. Effective hemodialysis and hemofiltration driven by an extracorporeal membrane oxygenation pump in infants with hyperammonemia. J Pediatr 1996;128:379-82.
13. Tuchman M, Mauer SM, Holzknecht RA, Summar ML, Vnencak-Jones CL. Prospective versus clinical diagnosis and therapy of acute neonatal hyperammonaemia in two sisters with carbamyl phosphate synthetase deficiency. J Inherit Metab Dis 1992;15:269-77.
14. Donn SM, Swartz RD, Thoene JG. Comparison of exchange transfusion, peritoneal dialysis, and hemodialysis for the treatment of hyperammonemia in an anuric newborn infant. J Pediatr 1979;95:67-70.

15. Sadowski RH, Harmon WE, Jabs K. Acute hemodialysis of infants weighing less than five kilograms. Kidney Int 1994;45:503-6.
16. Lettgen B, Bonzel KE, Colombo JP, Fuchs B, Kordass U, Wendel K, et al. Therapy of hyperammonemia in carbamyl phosphate synthetase deficiency with peritoneal dialysis and venovenous hemofiltration. [German]. Monatsschrift Kinderheilkunde 1991;139:612-17.
17. Vats A, Kashtan CE, Tuchman M, Mauer M. Hemodialysis catheter placement and recirculation in treatment of hyperammonemia. Pediatr Nephrol 1998;12:592-95.
18. Wong KY, Wong SN, Lam SY, Tam S, Tsoi NS. Ammonia clearance by peritoneal dialysis and continuous arteriovenous hemodiafiltration. Pediatr Nephrol 1998;12:589-91.
19. Brusilow SW, Valle DL, Batshaw M. New pathways of nitrogen excretion in inborn errors of urea synthesis. Lancet 1979;2:452-4.
20. Connelly A, Cross JH, Gadian DG, Hunter JV, Kirkham FJ, Leonard JV. Magnetic resonance spectroscopy shows increased brain glutamine in ornithine carbamoyl transferase deficiency. Pediatr Res 1993;33:77-81.
21. Maestri NE, McGowan KD, Brusilow SW. Plasma glutamine concentration: a guide in the management of urea cycle disorders. J Pediatr 1992;121:259-61.
22. Takahashi H, Koehler RC, Hirata T, Brusilow SW, Traystman RJ. Restoration of cerebrovascular CO2 responsivity by glutamine synthesis inhibition in hyperammonemic rats. Circ Res 1992;71:1220-30.
23. Willard-Mack CL, Koehler RC, Hirata T, Cork LC, Takahashi H, Traystman RJ, et al. Inhibition of glutamine synthetase reduces ammonia-induced astrocyte swelling in rat. Neuroscience 1996;71:589-99.
24. Batshaw ML. Sodium benzoate and arginine: alternative pathway therapy in inborn errors of urea synthesis. Prog Clin Biol Res 1983;127:69-83.
25. Green TP, Marchessault RP, Freese DK. Disposition of sodium benzoate in newborn infants with hyperammonemia. J Pediatr 1983;102:785-90.
26. Brusilow SW. Arginine, an indispensable amino acid for patients with inborn errors of urea synthesis. J Clin Invest 1984;74:2144-8.
27. Kline JJ, Hug G, Schubert WK, Berry H. Arginine deficiency syndrome. Its occurrence in carbamyl phosphate synthetase deficiency. Am J Dis Child 1981;135:437-42.
28. Brusilow SW, Batshaw ML. Arginine therapy of argininosuccinase deficiency. Lancet 1979;1:124-7.
29. Coude FX, Grimber G, Parvy P, Rabier D, Petit P. Inhibition of ureagenesis by valproate in rat hepatocytes. Role of N-acetylglutamate and acetyl-CoA. Biochem J 1983;216:233-6.
30. Gram L, Bentsen KD. Valproate: an updated review. Acta Neurol Scand 1985;72:129-39.
31. Kamoun P, Rabier D. Valproate-induced inhibition of urea synthesis. Lancet 1987;1:48.

The Journal of Experimental Biology 206, 2083-2087

doi:10.1242/jeb.00361

Review

The caveolar nitric oxide synthase/arginine regeneration system for NO production in endothelial cells

Larry P. Solomonson*, Brenda R. Flam, Laura C. Pendleton, Bonnie L. Goodwin and Duane C. Eichler

Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, FL 33612, USA

*Author for correspondence (e-mail: lsolomon@hsc.usf.edu)

Accepted 6 March 2003

Summary

The enzyme endothelial nitric oxide synthase (eNOS) catalyzes the conversion of arginine, oxygen and NADPH to NO and citrulline. Previous results suggest an efficient, compartmentalized system for recycling of citrulline to arginine utilized for NO production. In support of this hypothesis, the recycling enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL), have been shown to colocalize with eNOS in caveolae, a subcompartment of the plasma membrane. Under unstimulated conditions, the degree of recycling is minimal. Upon stimulation of NO production by bradykinin, however, recycling is co-stimulated to the extent that more than 80% of the citrulline produced is recycled to arginine. These results suggest an efficient caveolar recycling complex that supports the receptor-mediated stimulation of endothelial NO production. To investigate the molecular basis for the unique location and function of endothelial AS and AL, endothelial AS mRNA was compared with liver AS mRNA. No differences were found in the coding region of the mRNA species, but significant differences were found in the 5′-untranslated region (5′-UTR). The results of these studies suggest that sequence in the endothelial AS-encoding gene, represented by position –92 nt to –43 nt from the translation start site in the extended AS mRNA 5′-UTRs, plays an important role in differential and tissue-specific expression. Overall, a strong evidential case has been developed supporting the proposal that arginine availability, governed by a caveolar-localized arginine regeneration system, plays a key role in receptor-mediated endothelial NO production.

Key words: nitric oxide, eNOS, endothelial nitric oxide synthase, arginine, citrulline, arginine regeneration system, argininosuccinate synthase, argininosuccinate lyase, caveolae, nitric oxide production.

Introduction

Endothelial nitric oxide synthase (eNOS), the enzyme that catalyzes the production of NO from the amino acid arginine in endothelial cells, plays a key role in vasoregulation as well as in other important physiological processes such as angiogenesis. Impaired production of endothelial NO has been associated with hypertension, heart failure, hypercholesterolemia, atherosclerosis and diabetes (Govers and Rabelink, 2001; Vallance and Chan, 2001; Maxwell, 2002). Circulating effectors, such as bradykinin, bind to receptors on the luminal surface of endothelial cells, signaling the transient release of NO to the adjacent smooth muscle layer and resulting in relaxation of the vessel wall.

The signal for eNOS activation is a transient increase in intracellular calcium, which activates the enzyme through binding of a calcium–calmodulin complex (Ca–Cam). Endothelial NOS activation also occurs in response to shear stress (Govers and Rabelink, 2001; Maxwell, 2002). Consistent with the important physiological roles of eNOS, the enzyme appears to be subject to multiple modes of regulation, in addition to primary regulation through reversible Ca–Cam binding and activation. These include reversible phosphorylation and palmitoylation, substrate and cofactor availability, dimerization of enzyme subunits, intracellular translocation and protein–protein interactions (Govers and Rabelink, 2001). Several of these potential modes of regulation appear to be interrelated. As a component of caveolae, a subcompartment of the plasma membrane that serves to sequester proteins involved in cell signaling, eNOS may transiently interact with several different caveolar components. Previous work from several different laboratories has suggested that a diverse group of proteins, including calmodulin, caveolin-1, bradykinin B2 receptor, heat shock protein 90, argininosuccinate synthase (AS), argininosuccinate lyase (AL), Raf-1, Akt, extracellular signal-related kinase,

eNOS interacting protein, eNOS traffic inducer and unidentified tyrosine-phosphorylated proteins (Hellermann et al., 2000; Govers and Rabelink, 2001; Maxwell, 2002; Nedvetsky et al., 2002), may be transiently and functionally associated with eNOS.

A potential limiting factor for endothelial NO production is the availability of the substrate, arginine. Intracellular levels of arginine have been estimated to range from 100 μmol l^{-1} to 800 μmol l^{-1}, which is well above the K_m value of 5 μmol l^{-1} for cNOS (Harrison, 1997). Endothelial NO production can, nonetheless, be stimulated by exogenous arginine (Vallance and Chan, 2001). This phenomenon, termed the 'arginine paradox', suggests the existence of a separate pool of arginine directed to endothelial NO synthesis. As illustrated in Fig. 1, arginine has a number of metabolic roles in addition to NO production, including production of major metabolites such as urea, polyamines, creatine, ornithine and methylarginine derivatives. The observed stimulation of endothelial NO production by exogenous arginine suggests that the arginine directed to NO production may be segregated from bulk cellular arginine utilized for these other metabolic roles.

One possible site of control is at the level of arginine uptake. McDonald et al. (1997) showed that the CAT1 transporter, responsible for 60–80% of total carrier-mediated arginine transport into endothelial cells, colocalizes with eNOS in caveolae. They proposed that the arginine utilized by cNOS might, at least in part, be maintained by the CAT1 transporter. Another important mechanism for controlling the availability of arginine directed to NO production may be the regeneration of arginine from the other product of the eNOS-catalyzed reaction, citrulline. Hecker et al. (1990) initially demonstrated that citrulline, produced in the conversion of arginine to NO, can be recycled to arginine. A possible link between NO production and arginine regeneration from citrulline was subsequently established for other cell types (Nussler et al., 1994; Shuttleworth et al., 1995). This regeneration is catalyzed by the enzymes AS and AL, both of which also play an essential role in the urea cycle in liver. The potential importance of this regeneration system for endothelial NO production was supported by a report of two infants with a deficiency of AL who were shown to be hypertensive (Fakler et al., 1995). Upon infusion of arginine, the blood pressure of these infants decreased to near normal levels, suggesting a critical role for arginine regeneration in the regulation of systemic blood pressure. More recent evidence from DNA microarray analysis suggests an important role for the arginine regeneration system by clearly demonstrating significant and coordinate upregulation of AS-encoding gene expression in response to shear stress stimulation of endothelial NO production (McCormick et al., 2001). It was concluded that available arginine is a prerequisite for NO production and that in the absence of synthesis of additional eNOS, shear stress-induced increases in NO synthesis depend on an increase in synthesis of arginine from citrulline through increased AS expression. Although supplemental arginine can be beneficial in some cases (Wu and Meininger, 2002), in other cases it may lead to adverse effects owing to the multiple metabolic roles of arginine (Chen et al., 2003; Loscalzo, 2003).

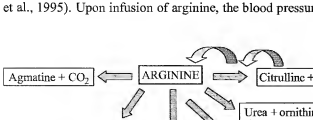


Fig. 1. Metabolic roles and fates of arginine. In addition to incorporation into protein, arginine serves as a metabolic precursor for several important metabolites, as indicated by the arrows. Also indicated is the two-step conversion of citrulline to arginine.

Recent work further supports the hypothesis that the arginine regeneration system, comprised of a caveolar complex that includes eNOS, AS and AL, plays an important, and most likely essential, role in the receptor-mediated production of NO by vascular endothelial cells.

Effects of exogenous arginine and citrulline on endothelial NO production

Endothelial NOS is localized in plasmalemmal caveolae. The localization of eNOS in this signaling subcompartment of the plasma membrane may have important implications with regard to the regulation and catalytic efficiency of eNOS (Everson and Smart, 2001; Shaul, 2002). We have recently found evidence for an efficient cycling of citrulline to arginine, raising the possibility of a channeling complex of eNOS and the enzymes of the citrulline–arginine cycle (AS and AL) localized in caveolae. Our initial research effort that led to this finding was designed to test the hypothesis that an intracellular pathway exists for the generation of methylarginines to regulate NO production in nitric oxide-producing tissues. The goal of this initial work was to determine the physiological significance of intracellular methylarginines as regulators of NOS activity. To examine the levels of endogenous methylarginines, we developed methods that allowed for the rapid and quantitative analysis (by HPLC) of arginine, citrulline and the methylarginines from endothelial cell extracts. There was no apparent change in levels of methylarginines following stimulation of endothelial cells with either bradykinin or the calcium ionophore A23187. In an attempt to raise intracellular methylarginine levels, and further test our hypothesis, we added citrulline, which we expected to inhibit dimethylarginine dimethylaminohydrolase, the enzyme that converts N^G-methylarginine or N^G,N^G-dimethylarginine to citrulline and monomethylamine or dimethylamine, respectively. The objective was to determine whether inhibition of the degradation of methylarginines would increase their intracellular concentrations and thereby inhibit NO production. To our surprise, stimulation of NO production by bradykinin was increased by the addition of citrulline, rather

than decreased, and there was no apparent change in methylarginine levels. To further examine the molecular basis for the stimulation of NO production by citrulline, we compared the effect of exogenous citrulline with the effect of exogenous arginine on NO production and levels of intracellular arginine following bradykinin activation. Surprisingly, added arginine did not cause as great an increase in endothelial NO production as did added citrulline. In addition, there was a much larger increase in intracellular arginine in response to exogenous arginine compared with exogenous citrulline. Added citrulline caused only a modest increase in intracellular arginine, while added arginine caused a substantial increase. Thus, there appeared to be no correlation between total intracellular arginine levels and endothelial NO production. To the best of our knowledge, this represents the first attempt to correlate NO production with the levels of intracellular arginine. Furthermore, the effects of arginine and citrulline on NO production appeared to be synergistic, since a combination of arginine and citrulline stimulated endothelial NO production more than did either arginine or citrulline alone (Flam et al., 2001). Since arginine has a number of potential metabolic fates, while citrulline has only one known metabolic fate (Fig. 1), the efficiency of NO production could be enhanced if a separate pool of arginine is maintained by endothelial cells. Recycling the product of the NOS-catalyzed reaction, citrulline, back to arginine *via* the enzymes of the arginine regeneration system, AS and AL, would maintain this separate pool. The pool of arginine used for NO synthesis would be essentially isolated from the bulk of intracellular arginine through the efficient operation of an arginine regeneration system. The apparent efficiency of the process suggests a channeling of intermediates and a compartmentalized complex of eNOS and enzymes of the arginine regeneration system. These results further support a model in which eNOS is localized together with this arginine regenerating system, and regulatory components, to ensure optimal efficiency of NO production and regulation without affecting other arginine-dependent cellular processes.

Caveolar localization of arginine regeneration enzymes with eNOS

Endothelial NOS is targeted by acylation to caveolae, where it interacts with caveolin-1 (Everson and Smart, 2001; Shaul, 2002). In liver cells, the arginine-generating enzymes AS and AL are associated with the outer mitochondrial membrane, reflecting the functional role of these enzymes in the production of urea (Cohen and Kuda, 1996). To test the model for a colocalization of AS and AL with eNOS, we used two different fractionation protocols for the purification of caveolae (Smart et al., 1995; Song et al., 1996). Both protocols generated a caveolar membrane fraction that was highly enriched in caveolin-1, eNOS, AS and AL (Flam et al., 2001). These results support the proposal that a separate pool of arginine, directed to NO synthesis, is effectively separated from the bulk of intracellular arginine through the functional localization of arginine regeneration enzymes and eNOS with plasmalemmal caveolae. A possible consequence of this functional association would be the channeling of intermediates through AS, AL and eNOS such that intermediates of the complex would not equilibrate with bulk intracellular arginine.

Degree of recycling

Cellular activity of eNOS has been estimated by measuring the rate of conversion of [^{3}H]arginine to [^{3}H]citrulline (Hardy and May, 2002). If recycling of citrulline to arginine is tightly coupled to NO production, this measurement would underestimate the cellular activity of eNOS. Estimating cellular activity of eNOS by measuring rate of production of NO (as the degradation product nitrite), on the other hand, should give a better estimate of cellular activity of eNOS. To test this hypothesis, and to estimate the degree of recycling of citrulline to arginine, we simultaneously measured the apparent rate of arginine-to-citrulline conversion and the rate of production of NO under both unstimulated and stimulated (addition of bradykinin) conditions. The ratio of these activities was close to one under unstimulated conditions. An increase in the ratio of NO produced to citrulline produced was approximately eight upon exposure of endothelial cells to agonist (B. R. Flam, D. C. Eichler and L. P. Solomonson, unpublished), indicating that recycling and NO production were costimulated. These preliminary results suggest an efficient caveolar complex for the regeneration of arginine directed to receptor-mediated production of NO in endothelial cells and an efficiency of greater than 80% for the recycling of citrulline to arginine under conditions of maximum stimulation of NO production. Although recycling of citrulline to arginine has been assumed to be important for conservation and efficient utilization of arginine, the degree of recycling relative to NO production has not, to the best of our knowledge, been quantified. Our results suggest that this recycling, especially under stimulated conditions, may play a more important role in endothelial NO production than previously recognized.

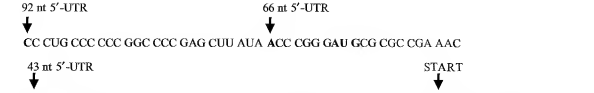


Fig. 2. Novel 5′ untranslated regions (UTRs) of endothelial argininosuccinate synthase mRNA.

Molecular basis for functional role and location of endothelial AS

In liver tissue, AS plays an essential role in urea synthesis and appears to be associated with the outer mitochondrial membrane (Cohen and Kuda, 1996). By contrast, endothelial AS appears to be the rate-limiting enzyme in the recycling of citrulline to arginine used for NO synthesis and is localized in caveolae (Flam et al., 2001). Immunoblotting experiments suggested small differences in subunit molecular masses and isoelectric points of endothelial AS compared with liver AS (B. R. Flam, D. C. Eichler and L. P. Solomonson, unpublished). We speculated that these differences could be due to a splice variant, but analysis of the coding sequence of AS mRNA indicated no differences between the mRNA from endothelial cells and liver (Pendleton et al., 2002). Because upstream and downstream untranslated regions (UTRs) of mRNA can influence regulation of gene expression, we carried out both 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE analysis to investigate possible differences in the UTRs. We found AS mRNA species with three different length 5′-UTRs in endothelial cells (Fig. 2). Only one of these products, the shortest 5′-UTR of 43 nt, was quantitatively expressed in liver. No significant variation was found in the 3′-UTR. The 5′-RACE analysis identified endothelial AS mRNA species with extended 5′-UTRs of 66 nt and 92 nt, in addition to a major 43 nt 5′-UTR AS mRNA (Fig. 2). Compositional analysis revealed that all three AS mRNA 5′-UTRs were enriched in G+C content (approximately 76%) and were likely to form complex and stable secondary structures. An upstream open reading frame (uORF) that was out-of-frame with the AS mRNA AUG start codon was detected in the 66 nt and 92 nt 5′-UTRs. RNase protection analysis (RPA) and real-time reverse transcriptase-PCR (RT-PCR) verified and quantified the differential expression of the extended 5′-UTR species relative to the major 43 nt 5′-UTR AS mRNA. Estimates from RPA of the amount of the 92 nt and 66 nt species, relative to the 43 nt species, were approximately 15% and 13%, respectively.

Features of mRNA UTRs, specifically uORFs, are regarded as important determinants of translational efficiency and may have important biological implications for the regulation of translation. We therefore designed experiments to determine to what extent the various 5′-UTRs of AS mRNA influenced translation. Translational efficiencies for the 66 nt and 92 nt AS 5′-UTR constructs were 70% and 25%, respectively, of the translational efficiency for the 43 nt 5′-UTR AS mRNA. Sequential deletions, starting with the 5′-terminus of the 92 nt 5′-UTR construct, resulted in a corresponding increase in translational efficiency, but the most pronounced effect resulted from mutation of the uORF, which restored translational efficiency to that observed with the 43 nt species. When the different AS mRNA 5′-UTRs, cloned in front of a luciferase reporter gene, were transfected into endothelial cells, the pattern of luciferase expression was nearly identical to that observed for the different 5′-UTR AS mRNAs in endothelial cells. These results suggest that a complex transcriptional/translational infrastructure exists to coordinate AS expression and NO production (Pendleton et al., 2002).

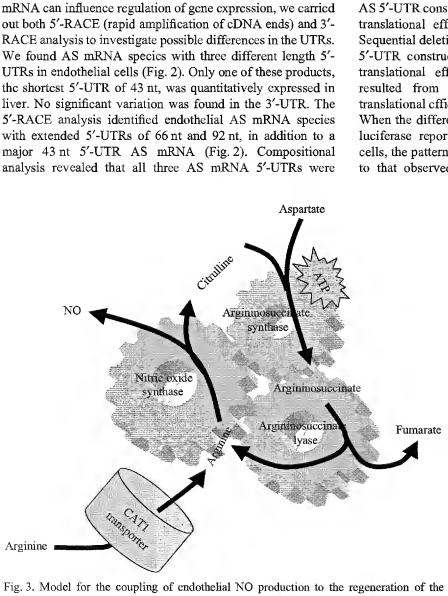


Fig. 3. Model for the coupling of endothelial NO production to the regeneration of the substrate, arginine, from the product, citrulline. Shown is the CAT1 transporter involved in arginine transport and the complex of argininosuccinate synthase and argininosuccinate lyase with endothelial nitric oxide synthase.

Model for coupling of arginine regeneration to endothelial NO production

A model depicting our view of the coupling of arginine regeneration to endothelial NO production through the compartmentalized complex of AS, AL and eNOS is shown in Fig. 3. This coupling may be largely 'disengaged' under unstimulated conditions but is 'engaged' and tightly coupled in response to agonists such as bradykinin. The molecular determinants and mechanisms involved in this coupling are not fully understood at this time. Based on our studies, and evidence from other labs, we believe the coupling of arginine regeneration to endothelial NO production is important for the overall regulation of endothelial NO production and may be essential for agonist-stimulated endothelium-dependent vasorelaxation.

The work described here was supported by the American Heart Association National Grant 9750222N, American Heart Association Florida Affiliate Grant 9950864V and the Mary and Walter Traskiewicz Memorial Fund.

References

Chen, J., Kuhlencordt, P., Urano, F., Ichinose, H., Astern, J. and Huang, P. L. (2003). Effects of chronic treatment with l-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knockout mice. *Arterioscler. Thromb. Vasc. Biol.* **23**, 97-103.

Cohen, N. S. and Kuda, A. (1996). Argininosuccinate synthetase and argininosuccinate lyase are localized around mitochondria: an immunocytochemical study. *J. Cell. Biochem.* **60**, 334-340.

Everson, W. V. and Smart, E. J. (2001). Influence of caveolin, cholesterol, and lipoproteins on nitric oxide synthase: implications for vascular disease. *Trends Cardiovasc. Med.* **11**, 246-250.

Fakler, C. R. (1995). Two cases suggesting a role for the L-arginine nitric oxide pathway in neonatal blood pressure regulation. *Acta Paediatr.* **84**, 460-462.

Flam, B. R., Hartmann, P. J., Harrell-Booth, M., Solomonson, L. P. and Eichler, D. C. (2001). Caveolar localization of arginine regeneration enzymes, argininosuccinate synthase, and lyase, with endothelial nitric oxide synthase. *Nitric Oxide* **5**, 187-197.

Govers, R. and Rabelink, T. J. (2001). Cellular regulation of endothelial nitric oxide synthase. *Am. J. Physiol. Renal Physiol.* **280**, F193-F206.

Hardy, T. A. and May, J. M. (2002). Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. *Free Radic. Biol. Med.* **32**, 121-131.

Harrison, D. G. (1997). Cellular and molecular mechanisms of endothelial cell dysfunction. *J. Clin. Invest.* **100**, 2153-2157.

Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E. and Vane, J. R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. USA* **87**, 8612-8616.

Heitermann, G. R., Flam, B. R., Eichler, D. C. and Solomonson, L. P. (2000). Stimulation of receptor-mediated nitric oxide production by vanadate. *Arterioscler. Thromb. Vasc. Biol.* **20**, 2045-2050.

Loscalzo, J. (2003). Adverse effects of supplemental L-arginine in atherosclerosis: consequences of methylation stress in a complex catabolism? *Arterioscler. Thromb. Vasc. Biol.* **23**, 3-5.

Maxwell, A. J. (2002). Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. *Nitric Oxide* **6**, 101-124.

McCormick, S. M., Eskin, S. G., McIntire, L. V., Teng, C. L., Lu, C. M., Russell, C. G. and Chittur, K. K. (2001). DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc. Natl. Acad. Sci. USA* **98**, 8955-8960.

McDonald, K. K., Zharikov, S., Block, E. R. and Kilberg, M. S. (1997). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the 'arginine paradox'. *J. Biol. Chem.* **272**, 31213-31216.

Nedvetsky, P. I., Sessa, W. C. and Schmidt, H. H. H. W. (2002). There's NO binding like NOS binding: protein–protein interaction in NO/eGMP signaling. *Proc. Natl. Acad. Sci. USA* **99**, 16510-16512.

Nussler, A. K., Billar, T. R., Liu, Z. Z. and Morris, S. M. (1994). Coinduction of nitric oxide synthase and argininosuccinate synthase in a murine macrophage cell line. Implications for regulation of nitric oxide production. *J. Biol. Chem.* **269**, 1257-1261.

Pendleton, L. C., Goodwin, B. L., Flam, B. R., Solomonson, L. P. and Eichler, D. C. (2002). Endothelial argininosuccinate synthase mRNA 5′-UTR diversity: infrastructure for tissue specific expression. *J. Biol. Chem.* **277**, 25363-25369.

Shaul, P. W. (2002). Regulation of endothelial nitric oxide synthase: location, location, location. *Annu. Rev. Physiol.* **64**, 749-774.

Shuttleworth, C. W., Burns, A. J., Ward, S. M., O'Brien, W. E. and Sanders, K. M. (1995). Recycling of L-citrulline to sustain nitric oxide-dependent enteric neurotransmission. *Neuroscience* **68**, 1295-1304.

Smart, E. J., Ying, Y. S., Mineo, C. and Anderson, R. G. (1995). A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. USA* **92**, 10104-10108.

Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M. and Lisanti, M. P. (1996). Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* **271**, 9690-9697.

Vallance, P. and Chan, N. (2001). Endothelial function and nitric oxide: clinical relevance. *Heart* **85**, 342-350.

Wu, G. Y. and Meininger, C. J. (2002). Regulation of nitric oxide synthesis by dietary factors. *Annu. Rev. Nutr.* **22**, 61-86.